

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.



# **A role of statins against *Listeria monocytogenes* and *Mycobacterium tuberculosis* infection**

**Suraj P. Parihar**

Thesis submitted to the University of Cape Town in fulfillment of the  
degree Doctor of Philosophy



Cytokines and Disease Group,  
International Centre for Genetic Engineering and Biotechnology  
(ICGEB), Cape Town Component and Institute of Infectious Diseases  
and Molecular Medicine (IIDMM), Division of Immunology, Faculty of  
Health Sciences, University of Cape Town,  
Cape Town, South Africa.

7<sup>th</sup> November 2011

*I dedicate this thesis to all my Best Friends,  
Mentors, Supervisors and specially my  
Parents and family, who stand with smiling  
faces in my absence on many occasions.*

## ACKNOWLEDGEMENTS

It is a pleasure to thank those who made this thesis possible. I owe my deepest gratitude to my supervisor, the best supervisor I could ask for, **Prof. Frank Brombacher** for allowing me to work in his competitive yet very supporting team and for his enthusiasm and guidance to achieve this milestone. He and his administrative team (**Ms. Dhuraiyah and Gloria**) have been very supportive since my first day on August 25<sup>th</sup> 2008.

It is an honor for me to work with **Dr. Reto Guler**, my co-supervisor for his guidance, understanding, inspiration and patience for establishing this project. His hunger for precision and scientific knowledge made him one of the highly regarded senior research scientist and the best instructor in the division. Working with him shaped my scientific insights by keeping science exciting to learn and carved me into independent thinker by providing me a unique opportunity to supervise students while still a graduate student. Above all he is very good friend and mentor. Since my arrival in Cape Town his family (including his mother) have welcomed me at their home for Christmas and many other occasions.

I would like to show my deepest gratitude to **Dr. Pawan Sharma** and **Dr. Kanury V. S. Rao** for shaping my interest in immunology of infectious diseases by providing me an opportunity to work in their research teams at ICGB-New Delhi, where I learned so many techniques and made life time friends. I am indebted to my M.Sc advisor and mentor at University of Rajasthan, **Dr. P.J. John** for his constant encouragement to achieve what I wanted in hard times. He is one of the important members who have significant impact on shaping my scientific interests.

During this work I have collaborated with many scientists for whom I have great regard, and I am indebted to **Dr. David Marais** for his expertise in the field of lipidemiology and critical inputs for the study. I would like to thank **Assoc. Prof. Muazzam Jacobs** and **Dr. Nasiema Allie** for their constant encouragement, support and providing an excellent BSL-3 facility.

I am also grateful to our very good friend and collaborator **Dr. Musa Mhlanga** for inviting me many times to work in his lab to analyze imaging data and good times with his very supportive team members. **Dr. Dirk M. Lang** for helping me to design some experiments and his excellent microscope facility and his immense knowledge in the field of microscopy.

My sincere thanks goes to medical nurses **Ms. Jenny Ross** (Lipid clinic) and **Ms. Phyllisty Smith Murphy** (Desmond Tutu HIV Foundation) for collecting blood samples for the study.

I would like to thank **Berenice Arendse** (then lab manager), **Wendy Green, Zenaria Abbas, Rayaana Fredericks, Fadwah Booley, Rosa Chang, Hylton, Faried, Babele, Lizette Fick, Marilyn Tyler** and **Zoe Lotz** for providing excellent technical support. A special thank goes to **Hiram Arendse, Rodney Lucas** and animal facility staff members for providing an excellent service during the course of study.

This thesis would have remained a dream had it not been support of my friends **Umeshree, Natalie, Claire, Anita, Toinette, Nasiema, Wendy, Dunja, Andrew, Hlumani, Alykhan, Tiroyane, Frank K, Bill, Jaisubhash, Phillipa, Sumaiyah and Roanne** for outings and fellow lab members for motivation, advice and most of all good times.

I owe special thanks to my friend **Liesel Smith** and her husband for being very caring and supporting, a friendship that deepened throughout. A special acknowledgement goes to my office mate **Ramona Hurdal**. She is true friend ever since we began to know each other. I would like to thank her family for being so nice to me, especially her mother for sending me lunch, when I wasn't in the mood of cooking whatsoever. Needless to say that she is the one who is very close to me. And her hugs will always missed and the laughs that made my years enjoyable and transformed into long-term memories. I also thank **Jackson Marakalala** and **Mark Barkhuizen** my other office mates for their ability to make us laugh by opera music and so on.

My sincere thanks go to **Claire** and **Umeshree** for revising English in this thesis. I would like to thank my friends from CSIR-Pretoria **Samantha, Robyn, Rethabile** and **Lionel** for being very nice host during my short visits. My deepest appreciation goes to my friends from ICGB-New Delhi and Jaipur. **Siddharth, Pavanish, Imran, Niladri, Hardeep, Javed, Kris, Sachin, Shashank, Prashant, Parveen and Richa** thank you all for good times, specially cricket during one of the crucial crossroad of my life.

And talking about Jaipur, I have special friends to thank, going back to my days of Masters degree. I am indebted to **Mr. J.N. Rawal** and his family to establish me in Jaipur and **Manendra, Vikas, Neel, Gunjan, Shaitan, Arun, Abishek, Mayank, Sandeep** and **Harish** for having lots of fun in Asthana Mansion. I wish to especially acknowledge the friendship with **Namrata** and **Neha** that began in Jaipur and grew throughout, whom I consider my best friends and will always cherish the times that we've had together and their families who have welcomed me at their home on innumerable occasions. The selfless support and encouragement that I received from **Neha**, I really wouldn't have been able to join in ICGEB-New Delhi.

Last but not the least; I owe my special thanks to my Grand parents, parents, brothers, wife and son for their unconditional love and support. They have lost a lot due to my research abroad. Without their understanding it would have been impossible for me to stay abroad and finish this work.

Importantly, I am also grateful to the ICGEB for awarding me pre-doctoral funding throughout the study.

### List of Publications

In addition to the work presented in this thesis, author has made significant contribution in following publications.

1. Statins counteract *Listeria*-induced phagosomal escape and *Mycobacterium*-inhibited phagosomal maturation and autophagy

**Suraj P. Parihar**, Reto Guler, Dirk M. Lang, Rethabile Khutlang, Musa Mhlanga, A. David Marais and Frank Brombacher (In Revision)

2. Simvastatin-induced autophagy result in protection against cutaneous leishmaniasis in mice

**Suraj P. Parihar**, Mary-Anne Hartley, Reto Guler, Ramona Hurdal, A. David Marais and Frank Brombacher (In preparation)

3. PKC $\delta$  regulates IL-12p40/p70 production by macrophages and dendritic cells, driving a type 1 healer phenotype in cutaneous leishmaniasis.

Guler R, Afshar M, Arendse B, **Parihar SP**, Revaz-Breton M, Leitges M, Schwegmann A, Brombacher F.

Eur J Immunol. 2011 Mar;41(3):706-15. Epub 2011 Feb 1.

4. Blocking IL-1 $\alpha$  but not IL-1 $\beta$  increases susceptibility to chronic *Mycobacterium tuberculosis* infection in mice.

Guler R, **Parihar SP**, Spohn G, Johansen P, Brombacher F, Bachmann MF.

Vaccine. 2011 Feb 1;29(6):1339-46. Epub 2010 Nov 18.

5. Human papillomavirus E6 and E7 oncoproteins as risk factors for tumorigenesis.

Ganguly N, **Parihar SP**.

J Biosci. 2009 Mar;34(1):113-23. Review.

**ABBREVIATIONS**

APCs	-Antigen presenting cells
β2-ME	-Beta-Mercaptoethanol
BMDM	-Bone marrow-derived macrophages
CFU	-Colony forming units
DCs	-Dendritic cells
DMEM	-Delbucco's Minimal Eagle's Medium
ELISA	-Enzyme-linked Immunosorbent Assay
FACS	-Fluorescence-activated cell sorter
FCS	-Fetal calf serum
FITC	-Fluorescein isothiocyanate
GM-CSF	-Granulocyte macrophage-colony stimulating factor
HDL	-High-Density Lipoproteins
H&E	-Haematoxylin and eosin
HRP	-Horseradish peroxidase
IFN-γ	-Interferon gamma
IL	-Interleukin
I.p	-Intraperitoneal
LDL	-Low-Density Lipoprotein
LDLR	-Low-Density Lipoprotein Receptor
LM	- <i>Listeria monocytogenes</i>
L929	- Murine Fibrosarcoma cell line for generating conditioned medium for BMDM
M Φ	-macrophages
MβCD	-Methyl-β-cyclodextrin
MHC II	-Major histocompatibility complex class II
Mtb	- <i>Mycobacterium tuberculosis</i>
ND	-Not detected
OD	-Optical density
PAMPS	-Pathogen-associated molecular patterns
PBS	-Phosphate buffered saline
NPP	-4-Nitrophenylphosphate
SEM	-Standard error of the mean
Th <sub>1</sub>	- T helper 1 cells, Th <sub>2</sub> - T helper 2 cells
VLDL	- Very Low-Density Lipoprotein
VLDLR	- Very Low-Density Lipoprotein Receptor



## Table of contents

<b>Abstract</b>	<b>1</b>
<b>Chapter 1: Introduction</b>	<b>3</b>
1. Innate immunity	4
1.1 Macrophage	4
1.2 Cytokines	10
2. Cholesterol	13
2.1 Cholesterol receptors	13
2.2 Lipid Rafts	14
2.3 Regulation of cholesterol in macrophages	14
3. Global use of statins	17
3.1 Prescription of statins and mode of action	17
3.2 Side effects of statins	20
4. Effect of statins on immune system	23
4.1 Pleiotropic effects	23
4.2 Infectious diseases	25
4.3 Inflammatory responses	26
4.4 Autoimmune diseases	27
5. Listeria	28
5.1 Listeriosis	28
5.2 Mouse model of listeriosis	30
5.3 Infection of macrophages with LM <i>in vitro</i>	30
6. Tuberculosis	33
6.1 Mouse model of tuberculosis	34
6.2 Infection of macrophages with <i>Mtb</i> <i>in vitro</i>	35
6.3 Phagolysosomes, an ideal niche for <i>Mtb</i>	36
7. Research covered in present study	39
7.1 Role of cholesterol in Listeriosis	39
7.2 Role of cholesterol in Tuberculosis	40
8. Observational studies of statins in humans	41
9. Rationale for the proposed study	42
10. Hypothesis	43
11. Objectives	43

12. References	44
<b>Chapter 2: Materials and Methods</b>	<b>55</b>
1. Mice	56
2. Ethics Statement	56
3. Bacterial cultures	
3.1 <i>Listeria monocytogenes</i> culture	56
3.2 <i>Mycobacterium tuberculosis</i> culture	57
4. <i>In vitro</i> macrophage culture and infections	
4.1 Bone marrow-derived macrophages (BMDM) culture	57
4.2 <i>Listeria monocytogenes</i> infection	57
4.3 <i>Mycobacterium tuberculosis</i> infection	58
4.4 Filipin staining in macrophages	58
4.5 Cholesterol content in macrophages	58
4.6 Cytokine ELISAs	58
4.7 Nitric oxide measurement	59
4.8 FACS analysis for MHC-II expression	59
4.9 MTT assay for cell viability and cytotoxicity	60
4.10 Phagocytosis assay	60
4.11 Immunofluorescence	60
4.12 Quantification of fluorescent images	61
5. <i>In vivo</i> infections	
5.1 Simvastatin treatment and LM infection in mice	61
5.2 Serum cholesterol measurement in mice	61
5.3 Tissue homogenates for enumeration of bacterial loads	62
5.4 Statin treatment and aerosol inhalation with <i>Mtb</i>	62
5.5 Monitoring of <i>Mtb</i> -infected mice	63
5.6 Histopathological examinations	63
6. Western blot analysis	
6.1 Preparation of macrophage whole-cell lysates	63
6.2 Protein content and Western blot analysis	63
7. Human mononuclear cells (PBMC) and macrophage culture	
7.1 Isolation of PBMC and monocyte-derived macrophages (MDM)	64
7.2 Intracellular growth of <i>Mtb</i> in human PBMCs and MDM	64
8. Statistical analysis	64

9. References	65
<b>Chapter 3: Results</b>	<b>66</b>
1. Optimization	
1.1 Intraperitoneal versus oral route of <i>L. monocytogenes</i> infection	67
1.2 Selection and dose of optimization of statins in mice	68
1.3 Effect of simvastatin on macrophage viability and proliferation	69
1.4 Simvastatin decreased intracellular cholesterol in macrophages	70
2. The role of statins during <i>Listeria monocytogenes</i> infection	
2.1 Statins had no major effect on extracellular growth of LM in culture	72
2.2 Simvastatin reduced intracellular growth of <i>L. monocytogenes</i> in macrophages	73
2.3 Increased IL-12p40, TNF- $\alpha$ and nitric oxide production by macrophages following simvastatin treatment	74
2.4 Simvastatin reduced IFN- $\gamma$ driven MHC-II expression in macrophages	76
2.5 Simvastatin had no major effect on phagocytic ability of macrophages	77
2.6 Statins decrease listerial growth in macrophage by interfering in LLO-dependent escape	79
2.7 Simvastatin decreased LM burden in mice	80
3. Role of statins during <i>Mycobacterium tuberculosis</i> infection	
3.1 The extracellular growth of <i>Mtb</i> is independent of statins in culture	82
3.2 Simvastatin reduced intracellular growth of <i>Mtb</i> in murine macrophages and human peripheral blood mononuclear cells (PBMC)	83
3.3 Statins promote maturation and induce autophagy	83
3.4 Statin mediated protection against <i>Mtb</i> in mice	86
3.5 Mononuclear cells and macrophages derived from patients on statin therapy are less susceptible to <i>Mtb</i> infection	88
4. References	90
<b>Chapter 4: Discussion</b>	<b>92</b>
1. Fig. Proposed model for statin-mediated protection against LM & <i>Mtb</i>	103

University of Cape Town

## List of figures and tables

### Figures

#### Chapter 1: Introduction

Figure 1: Targets of pattern-recognition receptors (PRR)	05
Figure 2: Different subtypes of macrophages and cytokine production	12
Figure 3: Regulation of cholesterol levels in macrophages	16
Figure 4: Cholesterol biosynthesis pathway	24
Figure 5: Recent outbreak of <i>Listeria</i> in United States	29
Figure 6: Listerial proteins internalin A (InlA) and InlB are host specific	31
Figure 7: Map showing Global distribution of tuberculosis	33
Figure 8: Intracellular life of <i>Mycobacterium tuberculosis</i>	38
Figure 9: Role of Cholesterol in <i>Listeria</i> infection	40
Figure 10: Role of Cholesterol in <i>Mtb</i> infection	41

#### Chapter 3: Results

Figure 1: Route of LM infection and LD <sub>50</sub> in mice	68
Figure 2: Effect of different doses and types of statins on LM burden in mice	69
Figure 3: Effect of cholesterol depletion on cell viability and proliferation	70
Figure 4: Simvastatin decreased intracellular cholesterol levels	71
Figure 5: Effect of simvastatin on growth of extracellular LM	72
Figure 6: Simvastatin decreased growth of LM in macrophages	73
Figure 7: Increased IL-12p40, TNF- $\alpha$ and nitric oxide production by macrophages following simvastatin treatment	75
Figure 8: Simvastatin decreased IFN- $\gamma$ driven MHC-II expression by macrophages following LM infection	77
Figure 9: Simvastatin had no effect on phagocytosis in macrophages	78
Figure 10: Simvastatin had no effect on growth of LLO-deficient LM in macrophages	79
Figure 11: LM burden is decreased in simvastatin-treated mice	81
Figure 12: Statin independent growth of <i>Mtb</i> in culture broth	82
Figure 13: Simvastatin decreased growth of <i>Mtb</i> in murine macrophages and human PBMC	83

Figure 14: Enhanced phagosomal maturation and autophagy following simvastatin treatment in murine macrophages	84
Figure 15: Phagosomal maturation and enhanced autophagy following simvastatin treatment in human PBMC	85
Figure 16: Decreased <i>Mtb</i> dissemination and reduced lung inflammation in statin-treated mice	87
Figure 17: Reduced growth of <i>Mtb</i> in human PBMC and MDM from statin-treated patients with familial hypercholesterolemia (FH)	88

## Chapter 4: Discussion

Figure 1: Proposed model on statin-mediated protection against LM and <i>Mtb</i> infection	103
--	-----

## Tables

### Chapter 1: Introduction

Table 1: Receptors of immune system	06
Table 2: Killing mechanism of activated macrophages	08
Table 3: Secretory factors released by activated macrophages	09
Table 4: Pharmacokinetic properties of various statins	18

# Abstract

University of Cape Town

**ABSTRACT**

Cholesterol has been shown to play important role in the pathogenesis and persistence of intracellular pathogens. Here, we modulate host cholesterol biosynthesis pathway using pharmacological agent statins, which are reversible inhibitors of HMG-CoA reductase enzyme. The aim of the study was to investigate the role of statins in inducing host protective responses against intracellular pathogens. We report reduced growth of *Listeria monocytogenes* (LM) and *Mycobacterium tuberculosis* (Mtb) in murine macrophages. We show prominent immunomodulatory activity induced by statins, mainly increased phagosomal maturation and autophagy resulting in decreased bacterial growth in macrophages. Subsequently, statin-treated mice showed decrease in bacterial loads, accompanied by reduced histopathology in the acute phase of infection during listeriosis and tuberculosis. Furthermore, we found decreased growth of Mtb in peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM) isolated from patients with familial hypercholesterolemia (FH) on statin therapy when compared to healthy subjects. Together, our results show that statins induces protection against Mtb in murine macrophages, mice and human mononuclear cells and monocyte-derived macrophages.



# **Chapter 1**

## **Introduction**

University of Cape Town

## **Introduction**

### **1. Innate Immunity**

The major role of the immune system is to combat infection elicited by foreign agents to reduce the ultimate damage to the host. There are two arms of the immune system, namely the innate immune response and the adaptive immune response. Innate immune responses are broad and uniform in all species and are present before the onset of infection. Professional phagocytes for example macrophages, dendritic cells and neutrophils along with the skin barrier and a range of antimicrobial compounds released by host cells are crucial for innate immunity which comprises the first line of defense. In contrast, the adaptive immune response comes into play only when host cells are challenged with antigens resulting in an increased specificity to a particular pathogen. Repeated encounters with that pathogen lead to an amplified secondary response as well as the extraordinary property of 'memory'.

#### **1.1 Macrophage**

Macrophages are one of the most important mediators of innate immune responses. This feature makes them a key contributor of the antimicrobial artillery of the innate immune response. These leucocytes are professional phagocytes, which bind to pathogens for ingestion, resulting in subsequent killing.

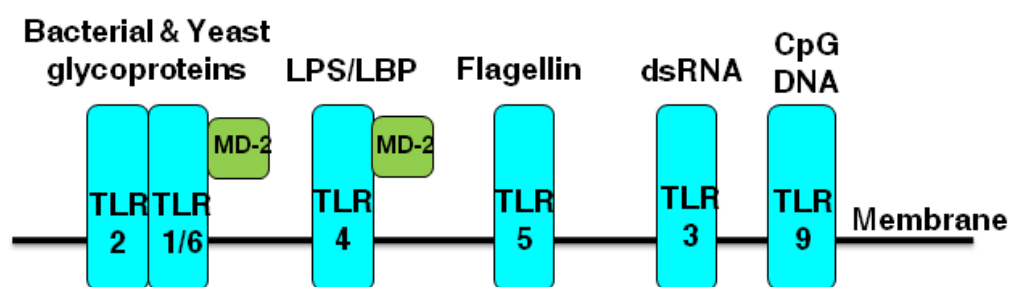
Macrophages differentiate from circulating blood monocytes. Resident macrophages differentiate in their local microenvironment and are widely distributed throughout the body. Activation and migration of other immune cells is greatly influenced by secretory products of macrophages that actively engage in endocytic and degradative processes. The elimination of intracellular pathogens depends on cellular interaction between macrophages and T lymphocytes. T cells recognize the processed antigen displayed on surface of macrophages via MHC II that in turn induce production of cytokines such as IFN- $\gamma$  by T cells and Natural Killer T cells (NKT). The secretion of IFN- $\gamma$  lead to production of IL-12 by macrophages that differentiate naïve T cells into T helper1 (Th1) cells that further enhance production of IFN- $\gamma$ , crucial for antimicrobial activity of macrophages.

The gene expression profile and cellular activities of macrophages are exceptionally varied under a homeostatic or host defense scenario. This heterogeneous response of macrophages is attributed to wide range of membrane receptors and secretory molecules, which empower them to interact with other leucocytes as well as virtually any other cell type in various tissues. Thus, macrophages are pivotal in regulating both innate and acquired immunity. Host viability is clearly dependent on the presence of macrophages.

### Receptors

Macrophages are highly skilled phagocytes capable of ingesting and subsequent digestion of ingested pathogens. To perform this vital function, these cells require crosstalk between pathogens and macrophage membrane receptors for downstream signalling and further activation of macrophages for host effector responses. This special type of phagocytosis is known as receptor-mediated endocytosis.

Scavenger receptors (SRs) and Toll-like receptors (TLRs) are mainly expressed on macrophages and dendritic cells (DCs) with the sole purpose of binding and internalization of both Gram-positive and Gram-negative bacteria. Apoptotic cells, which are recognized by SRs are also phagocytosed (Figure 1).



**Figure 1. Targets of pattern-recognition receptors (PRR).**

The extracellular location of PRR enables targeting of microbes or microbial components in extracellular fluids (blood and tissue fluids) leads to their lysis or removed via phagocytes. Binding of microbial components to PRR triggers signalling pathways that promotes inflammation and particularly in case of scavenger receptors (SRs) it induces phagocytosis. (Adapted and redrawn from Immunology- Kuby (5<sup>th</sup> edition) 2002 and S. Akira *et al.*, 2001, Nature Immunology 2:675).

TLRs are known as pattern recognition receptors (PRR), which recognize pathogen-associated molecular patterns (PAMP) and they play a crucial role in innate immune responses against invading pathogens (Lemaitre, Nicolas et al. 1996; Aderem and Ulevitch 2000). However, other receptors are also important and summarized in (Table 1).

Table 1. Receptors of immune System

Receptors of the innate immune system				
Receptor	Location	Target	Source	Effect of recognition
Complement	Bloodstream tissue fluids	Microbial cell wall components		Complement activation, opsonization
Mannose-binding lectin (MBL)	Bloodstream tissue fluids	Mannose-containing microbial carbohydrates	cell walls	Complement activation, opsonization
C-reactive protein (CRP)	Bloodstream tissue fluids	Phosphatidylcholine	microbial membranes	Complement activation, opsonization
LPS-binding protein (LBP)	Bloodstream tissue fluids	Bacterial lipopolysaccharide	LPS	Delivered to cell membrane LPS receptor
TLR2	Cell Membrane	Cell-wall components of gram-(+) bacteria LPS, Yeast cell-wall component	Zymosan	Attracts phagocytes, activates macrophages and dendritic cells. Induce secretion of several cytokines
TLR3	Cell Membrane	dsRNA	RNA viruses	Induce production of interferon, an antiviral

TLR4	Cell Membrane	LPS		cytokine  Attracts phagocytes, activates macrophages and dendritic cells. Induce secretion of several cytokines
TLR5	Cell Membrane	Flagellin	Flagella of gram-(+) & gram-(-) bacteria	Attracts phagocytes, activates macrophages and dendritic cells. Induce secretion of several cytokines
TLR9	Cell Membrane	CpG		Attracts phagocytes, activates macrophages and dendritic cells. Induce secretion of several cytokines
Scavenger receptors (many)	Cell Membrane	Many targets, gram-(+)/(-) bacteria  apoptotic host cells		Induce phagocytosis or endocytosis

Receptors of the adaptive immune system				
Antibody	B-cell membrane Blood, tissue fluids	Specific components of pathogen		Labelling of pathogen for destruction and removal
T-cell receptor	T-cell membrane	Protein or certain lipids of pathogen		Induction of pathogen-specific humoral and cell-mediated immunity

Adapted and redrawn from Immunology Kuby (2002)

TLR-induced activation of transcriptional activities results in the production of various cytokines to generate an inflammatory response, which attracts other macrophages and neutrophils to the site of infection. Therefore, TLRs link innate and acquired immunity (Akira and Takeda 2004). Activated macrophages eliminate internalized pathogens via either oxygen-dependent or oxygen-independent killing mechanisms (Table 2).

Table 2. Killing mechanisms of activated macrophages

Oxygen-dependent killing	Oxygen-independent killing
Reactive oxygen intermediates Superoxide anion ( $O_2^{\cdot -}$ ) Hydroxyl radicals ( $OH^{\cdot}$ ) Hydrogen peroxide ( $H_2O_2$ ) Hypochlorite anion ( $ClO^-$ )  Reactive nitrogen intermediates Nitric oxide (NO) Nitrogen dioxide ( $NO_2$ ) Nitrous acid ( $HNO_2$ )  Others Monochloramine ( $NH_2Cl$ )	Defensins  Tumor necrosis factor alpha   Lysozyme  Hydrolytic enzymes

Adapted and redrawn from Immunology-Kuby (2002)

Although, most of the ingested pathogens are degraded by macrophages, some of the processed pathogen antigens are also expressed on the macrophage membrane via the MHC-II endocytic pathway. This effectively activates T helper cells for humoral and cell-mediated immune responses. Macrophages activated in this manner release various secretory factors (Table 3).

Table 3. Secretory factors released by activated macrophages

Factors	Functions
Interleukin 1 (IL-1)	Promotes inflammatory response and fever
Interleukin 6 (IL-6) and TNF- $\alpha$	Promote innate immunity and elimination of pathogens
Complement proteins	Promote inflammatory response and elimination of pathogens
Hydrolytic enzymes	Promote inflammatory response
Interferon alpha (IFN- $\alpha$ )	Activates cellular genes, resulting in the production of proteins that confers an antiviral state on the cells
Tumor necrosis factor ( $\alpha$ )	Activation of NF- $\kappa$ B, MAPK pathways and induction of caspases, leading to apoptosis
IL-10	Immunoregulatory, down-regulate pro-inflammatory cytokine response
IL-12	Increase NKT cells and CD4/8 T cells for IFN- $\gamma$ production
IL-18	IFN- $\gamma$ inducing cytokine synergize with IL-12
GM-CSF, G-CSF & M-CSF	Promote inducible hematopoiesis

Adapted and redrawn from Immunology-Kuby (2002)

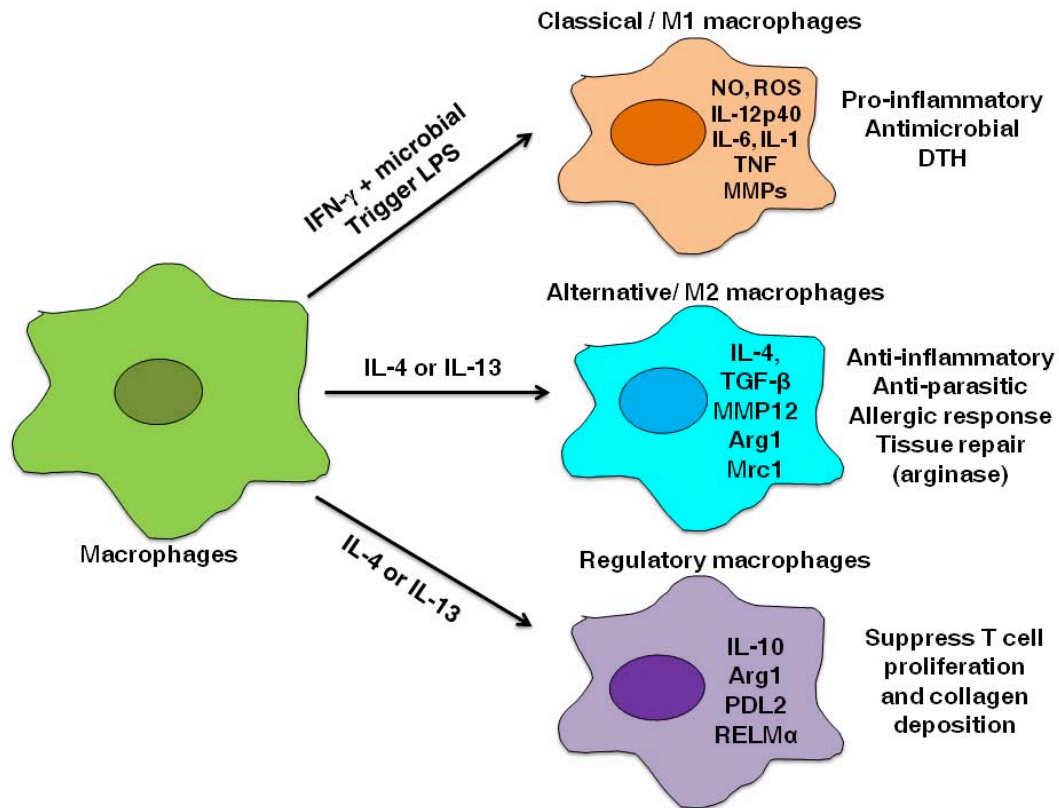
## 1.2. Cytokines

Cytokines are low-molecular weight secretory proteins released by various immune cells and are a category of signaling molecules that enhance cellular immune responses and are immunomodulating agents (Vilcek 1998). The cytokines include the interleukins (IL), lymphokines and cell signal molecules. The production of cytokines is transient and their action is autocrine, paracrine and endocrine (Kuby 2002). However, cytokine production is heterogeneous as any immune cell can produce it under given set of conditions.

Activated macrophages produce various cytokines in response to a stimulus, which are important inflammatory mediators. These cytokines include tumor necrosis factor alpha (TNF- $\alpha$ ), IL-1 and IL-6. Both IL-1 and TNF- $\alpha$  induce expression of endothelial adhesion molecules. For instance, induced expression of E-selectins via TNF- $\alpha$  results in selective binding of endothelial adhesion molecules to adhesion molecules on the surface of neutrophils (Kelly, Goldschmidt-Clermont et al. 1998). Integrins on circulating lymphocytes and monocytes are recognized and bind via ICAM-1 and VCAM-1 adhesion molecules expressed in presence of IL-1 (Zapolska-Downar, Siennicka et al. 2004). In addition to that, IL-1 and TNF- $\alpha$  act on macrophages to produce chemokines, required for the influx of neutrophils at the site of infection (Strieter, Kunkel et al. 1989). IL-12 further activates T cells to produce IFN- $\gamma$ , IL-2, TNF- $\alpha$  (Trinchieri 1997). Cytokines such as TNF- $\alpha$  and IL-1 or lipopolysaccharide (LPS) are major activator of Nuclear Factor-kappa B (NF- $\kappa$ B), which translocate to nucleus and induce transcription of target genes (IL-1, IL-6 and TNF- $\alpha$ ). Moreover, TNF- $\alpha$  and IFN- $\gamma$  activate macrophages to enhance their phagocytic activities and release hydrolytic enzymes in extracellular tissue spaces (Leenen, Canono et al. 1994). The systemic infections are characterized by enhanced production of IL-6, which activates the liver to produce acute phase proteins. This cytokine is secreted by macrophages as a pro-inflammatory marker of tissue damage or infection. It has been reported that IL-6 up-regulates expression of M-CSF receptor on monocytes to consume their own M-CSF, the interaction between IL-6 and M-CSF plays a crucial role in the differentiation of monocytes into macrophages (Chomarat, Banchereau et al. 2000).



There are distinct subset of macrophages, which regulates inflammation and wound healing. Because there is great overlap in surface markers expression between the different macrophage subsets, several macrophage subsets with distinct functions have been described. Classically activated (M1 macrophages) subset is well-characterized cells, which mediates defense of the host from various intracellular pathogens and have role in antitumor immunity. Activation of macrophages by TLR ligands (such as LPS) and by Th1 response (IFN- $\gamma$ ) exert pro-inflammatory and antimicrobial response such as production of nitric oxide (NO), reactive oxygen species (ROS), IL-1 and TNF. Alternatively activated macrophages (M2 macrophages) are activated by the Th2 cytokines such as IL-4 or IL-13. M2 macrophages have anti-inflammatory functions and regulate wound healing. M2 macrophages express arginase 1 (Arg1), mannose receptor CD206 (Mrc1) and matrix metalloproteinase 12 (MMP12). M2 macrophages are crucial for defense against helminthes, protozoan and fungal infections and have role in allergic responses (Gordon 2003; Murray and Wynn 2011). 'Regulatory' macrophages can secrete large amount of IL-10 in response to Fc receptor- $\gamma$  ligation (Sutterwala, Noel et al. 1998). The functions of M1, M2 and regulatory macrophages are summarized in figure 2.



**Figure 2. Different subsets of macrophages and cytokine production.**

Classical activation of macrophages is induced by Th1 cytokines such as IFN- $\gamma$  in combination with microbial stimulus (LPS). Classically activated macrophages have a pro-inflammatory and antimicrobial phenotype. Alternative and regulatory macrophages are activated by Th2 cytokines (IL-4/IL-13). Alternatively activated macrophages have an anti-inflammatory phenotype and produce high amounts of IL-10. (Adapted and redrawn from *Nature Reviews Immunology*, S. Gordon, 2003, 3:23 and *Nature Reviews Immunology*, Peter J. Murray and Thomas A. Wynn, 2011, 11:723).

## 2. Cholesterol

Cholesterol, an integral part of plasma membranes, is crucial for fluidity, viability and cellular proliferation and has a pivotal role in intracellular trafficking and signal transduction (Mukherjee and Maxfield 2004; Simons and Vaz 2004; Holowka, Gosse et al. 2005). It is also important precursor of steroid hormones and bile. A disorder in cholesterol levels which is highly regulated in membranes has severe consequences illustrated by Tangier disease, Nickmann-Pick type C and atherosclerosis (Maxfield and Tabas 2005). Cholesterol is synthesized by the endoplasmic reticulum (ER) and delivered to the membranes and other organelles via vesicular and non-vesicular transport (Maxfield and Wustner 2002; Vainio and Ikonen 2003; Soccio and Breslow 2004). Nearly 40% (van Meer 1989) to 90% (Lange, Swaisgood et al. 1989) of cellular cholesterol is present in the plasma membranes. Hence, cholesterol synthesis is dynamic process and finely tuned.

### 2.1 Cholesterol Receptors

Cells gain cholesterol by either *de novo* synthesis or receptor-mediated uptake of lipoproteins. To maintain homeostasis, cells also efflux cholesterol in circulation through High Density Lipoproteins (HDL), known as “good cholesterol” as it extract cholesterol from peripheral tissues. HDL does not stick to the walls of arteries and cholesterol is then transferred to the liver for the excretion. Cholesterol is delivered to the tissues via Low Density Lipoproteins (LDL) known as “bad cholesterol” they stick to the wall of arteries lead to development of plaques. LDL is strongly associated with the risk of heart diseases. Similarly, smaller amount of cholesterol can also be delivered to tissues via Very Low-Density Lipoproteins (VLDL) regarded as “very bad cholesterol” because it can deposit cholesterol on walls of the arteries which can increase the risk of non-fatal heart attack. The site of *de novo* synthesis of cholesterol is the ER where it's destined to the plasma membrane via a class of cellular proteins which bind to cholesterol known as caveolins, (Bloch 1965). The cellular uptake of the cholesterol is mediated by the receptors such as Low-density Lipoprotein Receptor (LDLR) (Brown and Goldstein 1976) and ApoB, which internalize LDL

bound cholesterol and hydrolytic pathways degrade the content of endosomes so as to deliver cholesterol into the cytosol and recycle receptors back to the cell surface for the next round of LDL uptake.

## **2.2 Lipid Rafts**

Lipid rafts are generally defined as cholesterol-rich membrane invaginations or caveolae or the insoluble membrane fraction including glycosphingolipid-enriched membrane domains (GEMs), detergent-resistant membranes (DRM) and the Triton-insoluble floating fraction (TIFF) of the membranes (Simons and Ikonen 1997; Rodgers and Zavzavadjian 2001). The phospholipids of the membrane bilayer are highly ordered in these regions thereby regulating signal transduction, whereas disordered domains are easily penetrated by water molecules, other small moieties and are susceptible to even weak detergents. Cholesterol that is recruited to the plasma membrane is confined to lipid rafts rather than being uniformly distributed throughout the membrane. The size of a single lipid raft is 50nm. Lipid-rafts mainly consists of cholesterol, sphingolipids and few proteins including GPI-anchored proteins (Varma and Mayor 1998).

## **2.3 Regulation of cholesterol in macrophages**

Cholesterol levels are important to maintain fluidity in cell membranes. Optimal levels are vital for cellular functions whereas sub-optimal or higher levels of cholesterol are detrimental to normal cellular physiology (Ikonen 2006). Membranes of macrophages need to be dynamic in order to efficiently internalize pathogens. As a result, fluidity in the pseudopodia is the ultimate requirement for membrane extension around particles to be engulfed and hence lipid levels are stringently controlled.

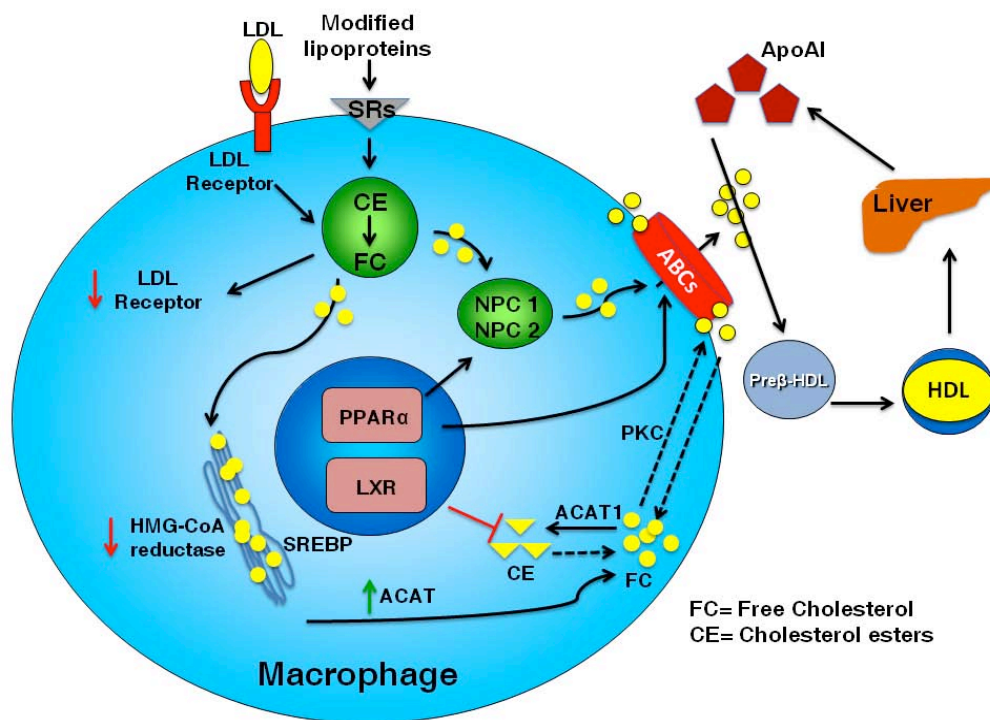
Macrophages express Low-Density Lipoprotein Receptors (LDLR) on its surface that bind to cholesterol containing low-density lipoproteins (LDL) which are subsequently internalized for lysosomal degradation. Following lysosomal degradation, cholesterol is released in cytosol and transported to the ER and the accumulation of cholesterol on the ER in turn decreases Hydroxy-methyl-glutaryl Coenzyme A (HMG-CoA) reductase activity via the sterol regulatory element-binding protein (SREBP) pathway as summarized (Figure 3). To stabilize excess cholesterol, cells increase the activity of acyl Co-A cholesterol

acyl transferase (ACAT), which stores excess cholesterol in tiny droplets of cholesteryl ester (CE) in the cytoplasm (Buhman, Accad et al. 2000; Chang, Li et al. 2009).

Macrophages also express the Very Low-Density Lipoprotein Receptor (VLDLR) which plays an important role in cholesterol metabolism via internalization of lipoproteins containing ApoE (Eck, Oost et al. 2005). VLDLR is well-conserved receptor present in human (Sakai, Hoshino et al. 1994) and mice (Gafvels, Paavola et al. 1994) and vastly expressed in heart, adipose tissue, brain, placenta and skeletal muscles (Takahashi, Kawarabayasi et al. 1992; Oka, Tzung et al. 1994). The expression of VLDLR on macrophages uncovered its potential role in foam cell formation that results in progression of atherosclerosis (Tiebel, Oka et al. 1999). The VLDLR expression is markedly increased on macrophages of rabbits feed on cholesterol diet (Hiltunen, Luoma et al. 1998). The mice deficient for VLDLR gene showed marked reduction in obesity and adipose tissue deposition (Goudriaan, Tacken et al. 2001). These results indicates physiological role of this receptor in delivering fatty acids in peripheral tissues. VLDLR also have been implicated in triglyceride metabolism via lipoprotein lipase (Lpl) activity (Yagyu, Lutz et al. 2002). The major breakthrough that established VLDLR as lipoprotein receptor, when adenovirus-mediated gene transfer correct hypercholesterolemia (Kobayashi, Oka et al. 1996) and reduced atherosclerosis in a murine model of familial hypercholesterolemia (FH) (Kozarsky, Jooss et al. 1996; Chen, Rader et al. 2000).

In addition to LDLR, macrophages also express scavenger receptors (SRs) that can internalize modified lipoproteins and stored in the cells as CE lead to lipid loading (Matsuura, Hughes et al. 2008). In cytoplasm CE is further converted into free cholesterol (FC). FC is exported via activation of transcription factors such as peroxisome-proliferator-activated receptors (PPAR $\alpha$ ) and liver X receptors (LXRs), which up-regulate Nickmann Pick cell (NPC) type 1/2 gene expression. Leading to enhance cholesterol levels in the membrane (Tabas 2000), which in turn efflux via the ABC (ATP-binding cassette) transporter ABCA1 pathway. In this way PPAR $\alpha$  and LXRs decrease the availability of

cholesterol to ACAT that prevent accumulation of cholesterol as CE in the cytoplasm (Argmann, Sawyez et al. 2003; Chinetti, Lestavel et al. 2003). The cholesterol efflux is mediated by apoAI that is induced by ABCA expression to pre $\beta$ -HDL or nascent HDL, once loaded with cholesteryl esters via lecithin cholesterol acyl transferase (LCAT) nascent HDL is converted to mature HDL (Rader 2006). HDL transports cholesterol from macrophages/peripheral tissues to the liver process known as reverse cholesterol transport (RCT). In liver cholesterol is secreted as such or secreted post conversion into bile acids (Chinetti, Fruchart et al. 2006). In addition, cholesterol efflux is also mediated by membrane diffusion and Protein Kinase C (PKC) has been implicated in this process (Li, Tsujita et al. 1997).



**Figure 3. Regulation of cholesterol levels in macrophages.**

The uptake of cholesterol enriched lipoproteins is mediated by SRs or LDLR receptors. Lysosomal hydrolysis release cholesterol in the cytoplasm, result in activation of sterol regulatory element-binding protein pathway (SREBP) in ER which suppress the gene expression of HMG-CoA reductase to decrease cellular *de novo* cholesterol biosynthesis. Transcription factors such as PPAR $\alpha$  and LXR which trigger efflux of cholesterol via NPC1/2 genes and ABCs (ATP-binding cassette) transporters. Hence, decrease the availability of free cholesterol (FC) to store in the form of cholesteryl esters (CE) by the action of ACAT enzymes. Free cholesterol also transported by the action of PKC via membrane diffusion. Extracellular cholesterol is then loaded into HDL and transported to the liver for subsequent bile acid synthesis or excreted. (Modified from Goldstein, J.L, Brown, M.S, 2009, *Arterioscler Thromb Vasc Biol*, 29:431, Chinetti, G et al., 2006, *Biochemical Society Transactions*, 6:1128 and Yokoyama, S. 1998, *Biochimica et biophysica Acta*, 1392:1).

### 3. Global usage of statins

#### 3.1 Prescription of statins and mode of action

In 2009, market sales of statins worldwide generated \$27bn in revenue (World Market 2011). Overwhelming success of statins is due to our current understanding of the low-density lipoproteins (LDL) pathway that contributes to coronary heart diseases and atherosclerosis. Targeting key enzymes of this pathway strikingly decreases LDL-cholesterol levels and cardiovascular events (Brown and Goldstein 1996). This is an important finding since heart diseases are the number one killer in humans. Hypercholesterolemia an asymptomatic disorder, results in higher serum cholesterol levels. An increased level of cholesterol, particularly LDL, delivers cholesterol in the peripheral tissues, resulting in the deposition on the endothelium. This is a major contributing factor to mortality in cardiovascular events when a level of LDL is more than 130mg/dL and total cholesterol is more than 200mg/dL.

Statins are the major success story in pharma industry because they reversibly target the rate-limiting enzyme, HMG-CoA reductase in the cholesterol synthesis pathway. Thus, reduces intracellular levels of cholesterol. Targeted inhibition of intracellular cholesterol synthesis leads to up-regulation of LDL receptors on the cell surface that increases uptake of LDL from the serum, which in turn reduces serum cholesterol level. Action of statins depends on the type of statin use as pharmacokinetic properties vary as summarized (Table 4).

Table 4. Pharmacokinetic properties of various statins

Parameter	Simva-	Atorva-	Fluva-	Lova-	Rosuva-	Prava-
	-statin					
<b>T<sub>max</sub> (Hr)</b>	1.3-2.4	2-3	0.5-1	2-4	3	0.9-1.6
<b>C<sub>max</sub> (ng/ml)</b>	10-34	27-66	448	10-20	37	45-55
<b>Solubility</b>	Lipophilic	Lipophilic	Lipophilic	Lipophilic	Hydrophilic	Hydrophilic
<b>T<sub>1/2</sub> (Hr)</b>	2-3	15-30	0.5-2.3	2.9	20.8	1.3-2.8
<b>Bioavailability (%)</b>	5	12	19-29	5	20	18
<b>Protein Binding (%)</b>	94-98	80-90	> 99	> 95	88	43-55
<b>Metabolism</b>	CYP3A4	CYP3A4	CYP2C9	CYP3A4	CYP2C9	Sulphation
<b>Metabolites</b>	Active	Active	Inactive	Active	Active	Inactive
<b>Transporter Protein Substrate</b>	Yes	Yes	No	Yes	Yes	Yes/No
<b>Urinary Excretion (%)</b>	13	2	6	10	10	20
<b>Fecal Excretion (%)</b>	58	70	90	83	90	71

Based on 40mg dose. C<sub>max</sub> = maximum concentration, T<sub>1/2</sub> =half-life and T<sub>max</sub> = time to maximum concentration. (Adapted from Bellost, S., et al, *Circulation*, 2004, 109:Supl III:50)



Reasons behind high cholesterol levels is classified into 3 categories by Centre for Diseases Control (CDC)

### 1. Conditions

#### *Age*

Cholesterol levels rises with increasing age. However, levels in females rise more than in males (Taylor and Agbedana 1983). Females have higher HDL levels than males at during their lifespan.

#### *Diabetes*

Other clinical manifestations like diabetes can also lead to high cholesterol levels. Malfunctions of insulin lead to diabetes because the body cannot remove sugar from circulation, thus leading to a rise in blood sugar levels.

### 2. Behavior

#### *Diet*

Diet with rich sources of saturated or trans fats, triglycerides or dietary cholesterol are one of the major source of increased cholesterol levels.

#### *Weight and lack of physical exercise*

Cholesterol rich diets can make one being overweight, which leads to higher LDL, a lower HDL level which shoots up total cholesterol levels. Synergistic with less exercise leads to increased weight.

### 3. Heredity

High cholesterol can be inherited from parents. The most common form of hereditary cholesterol is familial hypercholesterolemia (FH), a single gene point mutation that leads to a defect in the binding of LDL to LDLR resulting in elevated levels of serum cholesterol levels (Goldstein 1995). People with this disorder can have 4 times more cholesterol levels than healthy subjects. Such high levels of cholesterol may lead to early heart attacks and fatalities. It is noteworthy to mention that South Africa has one of the highest prevalence (1 in 100) of FH in the world among white Afrikaner, due to founder gene effect in emigrants from Europe (Seftel, Baker et al. 1980). Another genetic condition

known as familial dysbetalipoproteinemia or familial combined hyperlipoproteinemia, which is a defect in ApoE, results in an impaired clearance of VLDL, chylomicron and LDL from the circulation (Veerkamp, de Graaf et al. 2002; Ayyobi, McGladdery et al. 2003). This is leading to elevated levels of both cholesterol and triglycerides.

Patients with high cholesterol levels and FH are treated with cholesterol lowering drugs such as statins, along with little modification in diet, exercise and constant weight monitoring. Statin is also prescribed in conjunction with nicotinic acid in patients with high residual triglyceride levels, though they have reached the range of normal LDL levels (Vogt, Kassner et al. 2006).

Other potential benefits of statins have been reported and there is an ever-increasing interest in lipid independent uses of statins. The latent outcome of prolonged statin therapy on other diseases such as cancer and autoimmune diseases is of great interest. Since statins are widely used and well-tolerated drugs, their outcomes on other infections are likely to reduce global burden of certain clinical manifestations.

### **3.2 Side effects of statins**

Statins, the best-selling drugs in history to treat hypercholesterolemia, they are not devoid of side effects because they do interact with other drugs. Major clinical trials excluded subjects with statin intolerance and enhanced liver enzymes (ALT) concentrations in serum. However, constant report on adverse effects in statin users suggests that in rare conditions it may lead to muscle and liver damage.

#### **Myopathy**

All statins can cause muscle problems. Myopathy is defined as any disease associated with muscles. Myalgia is used for any muscle weakness or ache with no increase in creatine kinase (CK). Myositis is a condition with muscular pain and elevated CK. Rhabdomyolysis defined as severe muscular symptoms with 10 times higher levels of CK than normal upper levels. Rhabdomyolysis often begins with muscle pain and if left untreated, it can lead to loss of muscle cells, kidney failure and death. This mostly occurs when statins are used with other drugs

that have their own effect on muscles. Myalgia is the most common side effect and occurs in almost 7% of total statin users. The degree of myalgia increases with lipophilicity, dosage and the cholesterol lowering potency of statins. Among statins in the market, atorvastatin and simvastatin have the myotoxicity rate (Oskarsson 2011). Severe ailments like rhabdomyolysis and myositis are rare side effects of statins with an occurrence of 1.6 and 5 respectively per 100 000 individuals results similar to placebo groups. Cerivastatin has been withdrawn from the market due to its severe side effects leading to myositis and rhabdomyolysis (Gillett and Norrell 2011). An adverse effect on muscles is due to inhibition of protein prenylation and farnesylation. Ubiquinone (coenzyme Q10), a prenylated protein crucial for vital health shows decrease levels with the use of statins. Recent findings suggest that statin-mediated inhibition of Rab1 GTPase and impaired ER to golgi bodies (GB) transport results in toxicity in skeletal myofibers of rat (Sakamoto, Wada et al. 2011). The overall effects of statins are dose-dependent and have pro-apoptotic effects in muscles (Dirks and Jones 2006).

The risk of statin-mediated myopathy increased when administered with other drugs such as cyclosporine, erythromycin, verapamil etc. known to inhibit statin metabolism and prevent elimination. However, myopathy can be resolved after 2 months of discontinuing therapy. Individuals can restart with a lower dose or different statins thereafter.

#### Liver abnormalities

Statins are well tolerated, but despite the clear benefits and relative safety, the risk of liver failure is approximately 1 percent, which is similar to levels in subjects administered with placebo. Statins mediated increase in liver enzymes is clinically asymptomatic (less than 3 times of upper levels). Increased liver enzymes like alanine/aspartate transaminases are not true indicators of hepatic injury since transaminases can be higher with chronic viral hepatitis or non-alcoholic fatty liver disease. The best proper indicator of hepatic injury is the serum bilirubin level. However, the National Lipid Association recommends checking transaminases before initiation of statin therapy, 12 weeks post

initiation or increase in dose and periodically thereafter (McKenney, Davidson et al. 2006).

### Other affects

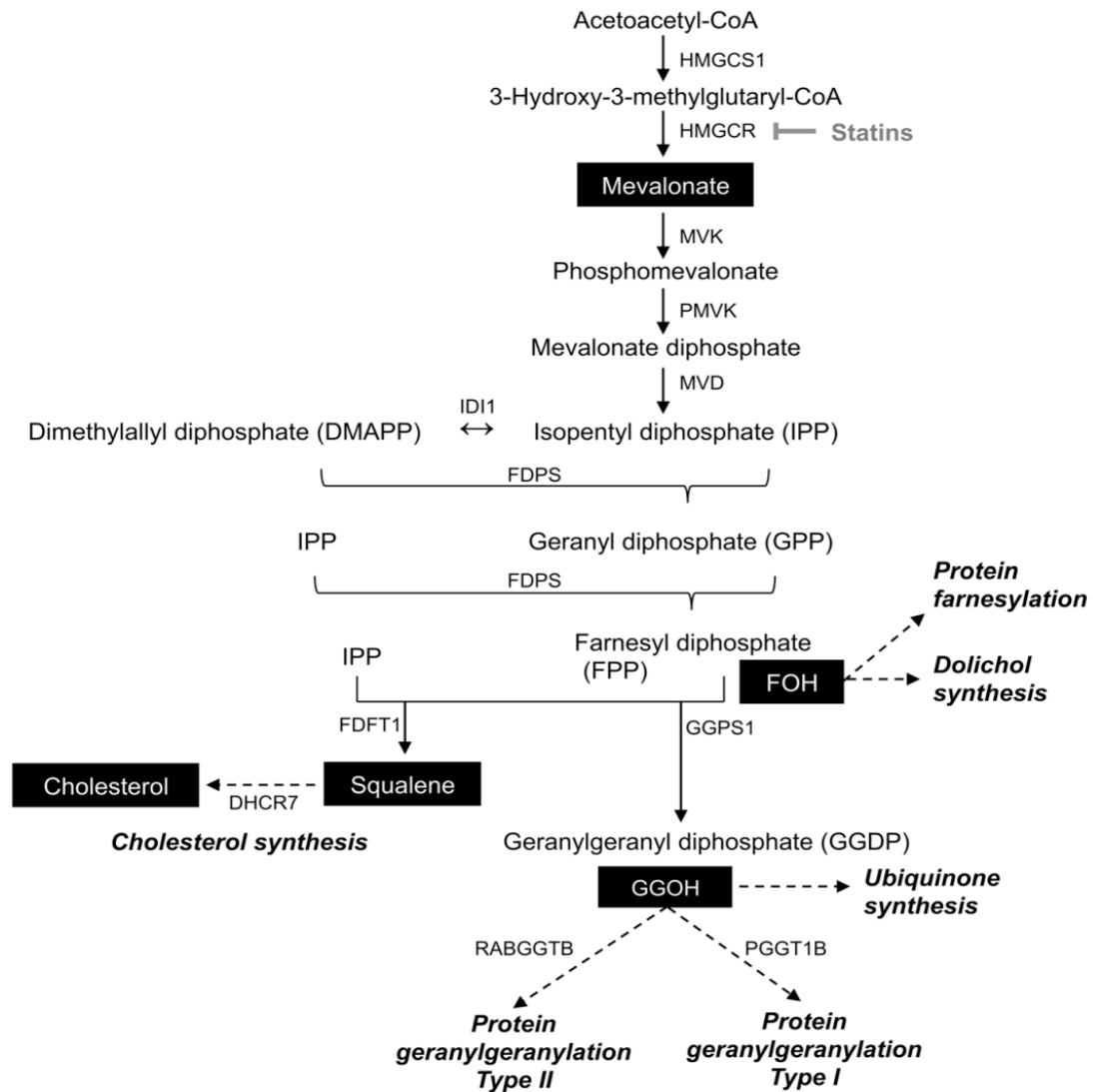
Most of the other side effects of statins emerge when taken with other drugs that interact with cytochrome P450. This interaction leads to inhibition of statin elimination from the circulation and leads to increased serum levels of statin causing adverse effects (David L. McClure, Robert J Valuck et al. 2007). Statins may have following side effects

- Nausea
- Irritability and short tempers
- Hostility
- Rapid loss of mental clarity
- Amnesia
- Kidney failure
- Diarrhea
- Cramping in the legs
- Inability to walk
- Problems sleeping
- Constipation
- Impaired muscle formation
- Erectile dysfunction
- Nerve damage
- Mental confusion
- Neuropathy

#### **4. Effect of statins on the immune system**

##### **4.1 Pleiotropic effects**

Increase in popularity of statins is undoubtedly due to its lipid-lowering effects. However, lipid independent effects of statins have been reported such as induction of endothelial nitric oxide which is important for vasodilation and inhibit platelet aggregates at site of plaques (Kaesemeyer, Caldwell et al. 1999). Statin-mediated increased bone morphogenic protein-1 (BMP-1) leads to new bone formation, a potential application of statin in treatment of osteoporosis. Besides bone formation, statins also suppress bone-eating osteoclast cells (Mundy, Garrett et al. 1999; Ayukawa, Yasukawa et al. 2009). Other lipid independent activities of statins include increased activity of endothelial nitric oxide synthase (eNOS)-dependent Akt kinase that promotes formation of new blood vessels (angiogenesis), a benefit for ischemic tissues (Kureishi, Luo et al. 2000). More recently, benefits of statins have been implicated in cancer via inhibition of proliferation and invasion of cancer cells (Glynn, O'Sullivan et al. 2008). Pleiotropic effects of statins have gained much attraction recently, as some benefits from statins are independent of lipid lowering abilities. These beneficial effects could be attributed to inhibition of protein farnenylation and prenylation, an intermediate arm of the mevalonate pathway crucial for post-translational modifications of proteins (Figure 4). Statins have also been shown to modulate cytokine-mediated induction of cell adhesion molecules (CAMs) via G protein, which is regulated by protein prenylation (Sadeghi, Collinge et al. 2000). However, these effects are reversible and discontinue when statin treatment is ended.



**Figure 4. Mevalonate-mediated cholesterol biosynthesis pathway.**

Statins inhibits HMG-CoA reductase to prevent synthesis of mevalonate and in turn cholesterol. (Adapted from Plos Biology, Mathieu Blanc, *et.al*, 2011)

Elevated levels of serum high-sensitivity C-reactive protein (hs-CRP) is an inflammatory biomarker and an early indicator of coronary heart diseases. This acute phase protein is released by the liver in response to IL-6 and indicates minor systemic inflammation. Statin therapy reduces plasma concentrations of hs-CRP independent of LDL cholesterol (Liao and Laufs 2005). Hs-CRP in conjunction with LDL-cholesterol accumulates within plaques, leading to progression of atherosclerosis. More recently, it has been shown that topical application of statins reverses genetic hair growth defects in mice caused by accumulation of HMG-CoA reductase protein in hair follicles (Evers, Farooqi et al. 2010).

## 4.2 Infectious Diseases

Beneficial effects of statins on infectious diseases are well documented in a variety of bacterial, viral and protozoan infections.

### Bacterial diseases

In mouse model, cerivastatin and simvastatin increased survival of lipopolysaccharide-induced sepsis in mice (Ando, Takamura et al. 2000; Merx, Liehn et al. 2004). Similarly, growth of *Salmonella enterica* was reduced in a murine macrophage cell line and in mice following treatment with lovastatin and atorvastatin respectively (Catron, Lange et al. 2004). Moreover, mice administered with simvastatin were also able to control growth of *Chlamydia pneumoniae* (Erkkila, Jauhiainen et al. 2005). In contrast, recent findings in macrophages infected with opsonized *Staphylococcus aureus* showed impaired killing following simvastatin treatment due to inhibition of protein prenylation (Benati, Ferro et al. 2010).

### Viral diseases

The role of statins in viral diseases is extensively published. Recent reports show that, dengue virus replication was inhibited in human PBMCs following treatment with lovastatin (Rothwell, Lebreton et al. 2009). Statin therapy leads to a decrease in HIV-1 viral loads and increase in CD4 counts in human PBMCs and chronically HIV-1 infected patients (del Real, Jimenez-Baranda et al. 2004). In mouse models, Epstein–Barr virus (EBV)-induced tumor formation in SCID mice was reduced following treatment with simvastatin resulting in increased survival (Katano, Pesnicak et al. 2004). More recently, simvastatin treatment resulted in decreased viral loads of mouse cytomegalovirus (CMV) in a variety of organs (Blanc, Hsieh et al. 2011).

### Protozoan diseases

With regards to protozoan parasites such as *Leishmania* promastigotes, cholesterol depletion by lovastatin reduced infection in peritoneal macrophages (Rub, Dey et al. 2009) and growth of *L. donovani* decreased in macrophage cell

line following M $\beta$ CD treatment (Pucadyil, Tewary et al. 2004). Cholesterol sequestration of erythrocytes by M $\beta$ CD reduced *Plasmodium* entry and infection (Lauer, VanWye et al. 2000). In line with protozoan parasites, the replication of *Toxoplasma gondii*, a parasite that acquires cholesterol by harnessing host LDLR mediated endocytosis decreased after synchronized inhibition of endogenous cholesterol by mevastatin and exogenous cholesterol by lipid deficient serum (Coppens, Sinai et al. 2000).

### 4.3 Inflammatory responses

Statins induce anti-inflammatory effects and this may play an important role as an additional mechanism involved in the statin-mediated beneficial effects. Potential anti-inflammatory effects of statins have been shown on monocytes of chronic pre-dialysis patients with marked reduction in IL-6 and IL-8 has been observed including inflammatory markers such as C-reactive protein (CRP) (Panichi, Paoletti et al. 2006). In contrast, statins have no mortality benefits in patients with end-stage renal diseases (ESRD) or uremia where mortality is due to cardiovascular ailments, as a result of excessive accumulation of toxic metabolites in circulation (Karumanchi and Thadhani 2010). Statins are inhibitors of T and B cell proliferation in vitro (Lawman, Mauri et al. 2004; Blank, Schiller et al. 2007). Statins also inhibit monocytes, negatively regulate chemokine receptors on T and B cell surfaces and induce some pro-inflammatory cytokines, indicating their immunomodulatory capabilities (Neuhaus, Strasser-Fuchs et al. 2002). Multiple organ dysfunction syndrome (MODS) is caused by consecutive failure of organs after onset of sepsis or cardiogenic shock. Statin therapy in MODS patients leads to 28 days survival benefits possibly by reducing inflammatory responses and increasing activity of vagal nerve to heart, lung, liver and upper/lower abdomen (Schmidt, Hennen et al. 2006).



#### 4.4 Autoimmune diseases

In addition to lowering lipids, statins have immunomodulatory and anti-inflammatory capabilities that are not only essential for cardiovascular diseases but also in the context of autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (Allan, Broady et al. 2008). The role of simvastatin has been reported in suppression of collagen-induced arthritis by reduction of Th1 and pro-inflammatory responses (Leung, Sattar et al. 2003). Given the modest toxicity associated with atorvastatin, this statin has been implicated in delivering protection against graft-versus host diseases in transplants by polarization of Th2 responses and suppression of Th1 polarization along with down regulation of MHC II and co-stimulatory molecules on antigen presenting cells (APCs) of recipients (Zeiser, Youssef et al. 2007). Statins do possess anti-inflammatory and inhibitory effects on acute phase proteins which could be beneficial in combating systemic lupus erythematosus (Wierzbicki 2001). Statins positively regulate type1 diabetes mellitus by preventing self-reactive T cells and established sugar levels in mice developing diabetes (Brumeanu, Goldstein et al. 2006). However, translation of statins in managing human autoimmune diseases is still at its infancy.

## 5. Listeria

Listeria is one of the most widely used organisms to study host immune responses due to its contribution to cell specific responses and its short course of infection (Pamer 2004). Here, we studied listeriosis as a short model to investigate potential outcome on tuberculosis infection, a long-term bacterial disease. Both share a common feature of infecting macrophages. Despite this, however, the immune evasion mechanisms vary to a large extent in these pathogens.

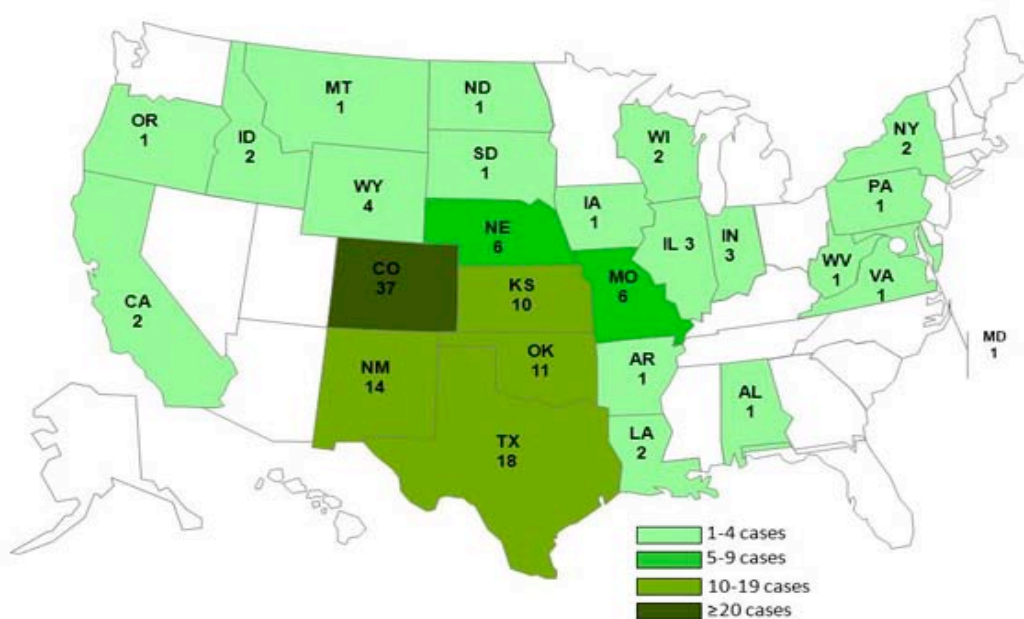
### 5.1 Listeriosis

Listeriosis is a food-borne infectious disease caused by *Listeria monocytogenes* an intracellular, gram-positive bacillus, 0.4-1.5 µm in size that thrives in all natural habitats. The natural niche of the genus *Listeria* is understood to be in decomposing plant matter where they live as saprophytes. They do not form spores, have no capsule and are motile at 10-25°C. The genus *Listeria* includes six species and two of these *L. monocytogenes* and *L. ivanovii* are potentially pathogenic. The clinical symptoms of listeriosis include gastroenteritis, encephalitis, and mother-to-fetus infections leading to septicemia, meningitis and spontaneous abortions respectively.

In 1924 *Listeria* was discovered by E. G. D Murray, R. A. Webb and M. B. R Swann as the etiological agent of septicemia in rabbits and guinea pigs. The organism *Listeria* was named *Listerella hepatolytica* after British surgeon Joseph Lister. The first case of human listeriosis was diagnosed in Denmark in 1929. However, in 1921 the first recorded culture of *Listeria monocytogenes* was isolated in France from a meningitis patient (Dumont 1921). In 1983 the series of epidemic outbreaks of listeriosis in North America and Europe established *Listeria* as an important food borne pathogen. *Listeria* infects humans by consumption of contaminated food products including soft cheese, sausages, patties, dairy products, smoked fish, salads and in general industrially produced, refrigerated ready-to-eat food products that do not have to be cooked or re-heated. *Listeria* occurs ubiquitously in rural environments and contaminates all raw materials and plants products used for industrially processed food. *L. monocytogenes* is

well armed to survive in food processing technologies. The ability of listeria that makes it a major threat to food industries attributed to its adeptness to high concentration of salts, comparatively low pH and is able to proliferate at refrigerated temperatures. Though, listeria infection is relatively rare but often-severe disease with high fatality rate of 20-30% among reported cases.

Repeated outbreaks of Listeriosis remind it is more deadly than well-known pathogens such as *Salmonella* and *E.coli*. For example, in 2000 a Listeriosis outbreak in France claimed the life of 7 individuals and 26 infected (WHO 2000). In 2008, the widespread outbreak of Listeriosis from Maple leaf food left 22 people dead and 57 infected in Canada (CFIA 2009). More recently, multistate outbreak of Listeriosis in the State of Colorado caused by contaminated Cantaloupes (Rock melon) lead to 28 deaths and 133 people infected in US (Figure 5), one of the worst in decades (CDC 2011).



**Figure 5. Recent outbreak of listeriosis in United States of America.**

On October 24, 2011, a total of 133 persons infected with any of the four outbreak-associated strains of *Listeria monocytogenes* have been reported to CDC from 26 states. All illnesses started on or after July 31, 2011. The number of infected persons identified in each state is as follows: Alabama (1), Arkansas (1), California (2), Colorado (37), Idaho (2), Illinois (3), Indiana (3), Iowa (1), Kansas (10), Louisiana (2), Maryland (1), Missouri (6), Montana (1), Nebraska (6), New Mexico (14), New York (2), North Dakota (1), Oklahoma (11), Oregon (1), Pennsylvania (1), South Dakota (1), Texas (18), Virginia (1), West Virginia (1), Wisconsin (2), and Wyoming (4).

The ability of *Listeria* to infect any cell type and cell-to-cell transmission without being exposed to the extracellular environment makes it an extremely successful and well-armed pathogen.

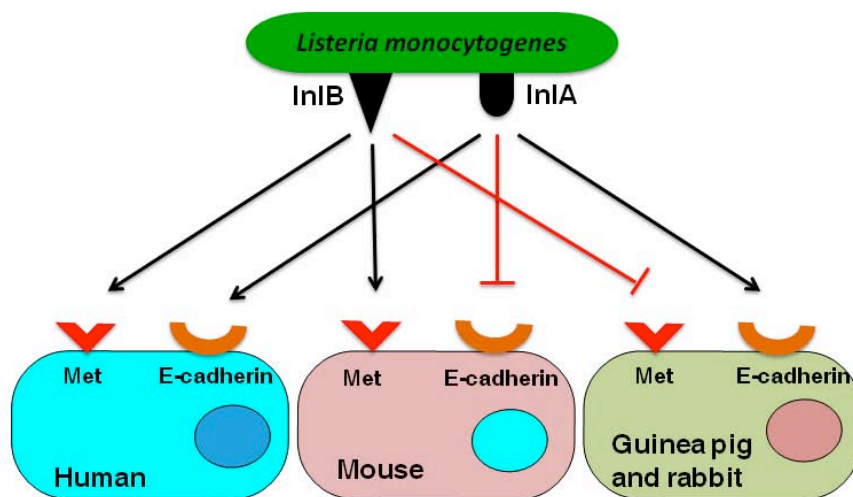
## 5.2 Mouse model of Listeriosis

Despite, Listeriosis is being of rare occurrence it has been one of the most extensively studied infection model especially for the study of innate immunity (Unanue 1997; Unanue 1997). LM can infect any cell type but this bacterium has a peculiar affinity to the meninges causing fatal meningitis. Initial exposure to LM is controlled at the cellular level by a strong pro-inflammatory innate immune response orchestrated by neutrophils, macrophage (Conlan and North 1994) and NKT cells. At the site of infection LM is internalized by macrophages (day1), which in turn release IL-12 and TNF- $\alpha$ , which further stimulates production of IFN- $\gamma$  by NKT cells. The production of IFN- $\gamma$  and TNF- $\alpha$  is required for essential primary defense against listeria infection (Pfeffer, Matsuyama et al. 1993; Rothe, Lesslauer et al. 1993; Harty and Bevan 1995). Activation of macrophages also secretes nitric oxide (NO), a key microbicidal factor that can kill both intracellular and extracellular pathogens effectively (Shiloh, MacMicking et al. 1999; Serbina, Salazar-Mather et al. 2003). Further influx of macrophages at the site of infection promotes the formation of granulomatous lesions. Innate immune response declines and recruitment of T cells (Th1 and cytotoxic T cells) at the site of infection following 6-7 days leads to complete eradication of pathogen in host, mediated by the adaptive arm of the immune system (Kaufmann, Follows et al. 1992). Apart from understanding innate immune responses, LM infection model is far more widely studied to investigate adaptive immune responses in host defense (Mackaness 1962).

## 5.3 Infection of macrophages with *Listeria monocytogenes* *in vitro*

In addition, LM is the most studied pathogen in the context of host-pathogen cell biology. Mostly LM are encountered by professional phagocytes namely macrophages and dendritic cells (Westcott, Henry et al. 2010), which passively internalize bacilli. The phagocytosis of LM can be mediated by the opsonin-dependent pathway, which is facilitated by Fc gamma receptors (Fc $\gamma$ R) and

CR3/C1qR complement receptors (Drevets, Leenen et al. 1993; Kolb-Maurer, Pilgrim et al. 2001). In contrast, the opsonin-independent pathway involves recognition of lipotechoic acid by the scavenger receptor (SR) on macrophages (Dunne, Resnick et al. 1994). LM also exploits its  $\alpha$ -D-galactose to invade dendritic cells. However, LM can also infect non-professional phagocytes by the bacterial proteins internalin A or B (InlA, InlB), which induce binding via to receptors on the surface of host cells. The majority of non-phagocytic cells express InlB receptors that mediate bacterial entry by actin rearrangement and the Met-signaling pathway. Met is a protein tyrosine kinase that is expressed by all cells of epithelial origin and the endogenous ligand of this receptor is the hepatocyte growth factor (HGF), which is produced by all cells of fibroblast and stromal origin. On the other hand, very few cell types express InlA where entry is mediated by epithelial cadherins (E-cadherins) and actin rearrangement. The engagement of receptors with listeria initiate downstream signal transduction which involves activation of kinases, lipid metabolism and actin remodeling resulting in internalization of LM (Jutras and Desjardins 2005). It is noteworthy to mention that, LM is internalized by a host specific manner (Figure 6).



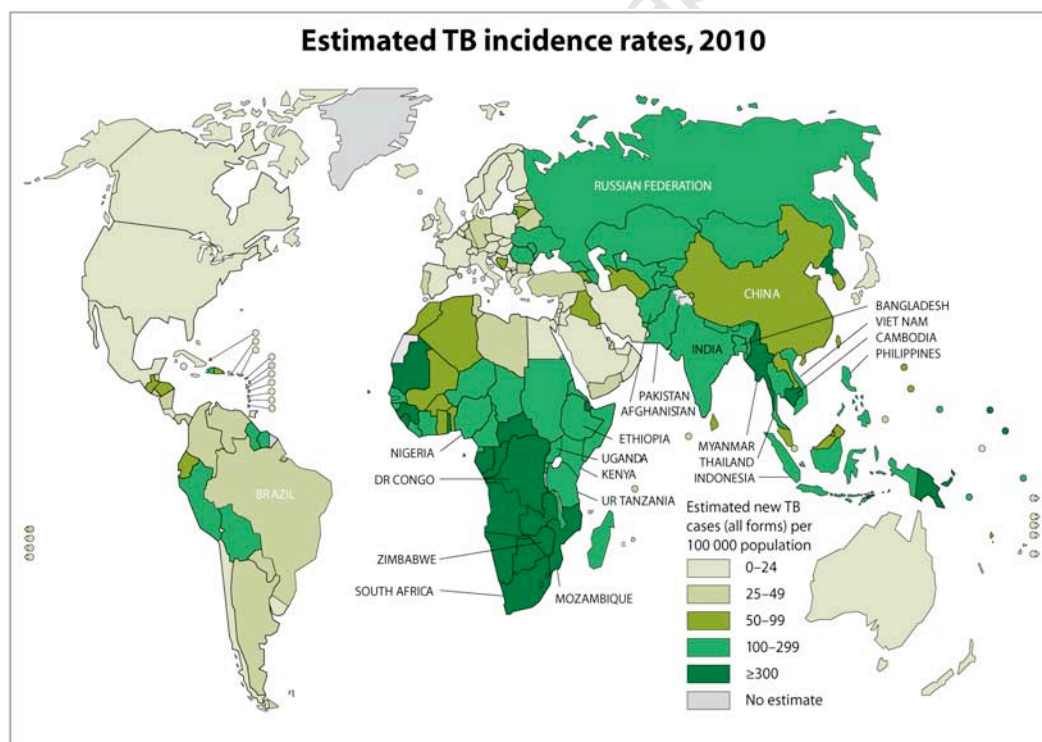
**Figure 6. Listerial proteins internalin A (InlA) and InlB are host specific.**

LM can bind and get entry into human host via both InlA and InlB, which induce epithelial cadherin (E-cadherin) and Met pathway respectively. Whereas entry in murine cells a single amino-acid change in E-cadherins inhibit it's binding to InlA. Similarly, InlB cannot activate Met pathway in guinea pigs and rabbit in an undefined manner. (Adapted and redrawn from M. Hamon et al., 2006, Nature Review Microbiology, 4: 423).

Once internalized, LM grows rapidly in murine macrophages and most of the internalized bacilli are killed during the first hour of infection. However, 14% of the internalized bacteria escape to cytoplasm during the first hour (de Chastellier and Berche 1994), which is attributed to the unique feature of LM to escape phagosomes by secreting the pore-forming cytolysin known as listeriolysin O (LLO). Listeriolysin is a cholesterol-dependent family of cytolysins, which inserts into the vacuolar membrane by binding cholesterol and forms pores that results in the phagosomal membrane disruption (Mengaud, Chenevert et al. 1987) and in addition it also express two phospholipases, PlcA and PlcB which help bacteria to escape into the cytoplasm, a strategic move to evade phagosomal degradation. Once, in the cytosol LM replicates quickly and bacterial protein ActA (Kocks, Gouin et al. 1992) which hijack host actin machinery to polymerize results in cell-to-cell transmission via actin comets/tails (Tilney and Portnoy 1989) without extracellular exposure. This is an efficient way to escape the humoral immune response (Cossart, Vicente et al. 1989; Dabiri, Sanger et al. 1990). The transmission of LM to adjacent cells leads to its capture in a double-membrane vacuole. LM expresses an enzyme lecithinase, an extracellular phosphoryl-choline phospholipase C (PlcB) and a secretory zinc metalloproteinase to escape the secondary vacuole of the newly invaded cell (Vazquez-Boland, Kocks et al. 1992). Then, LM secretes LLO that leads to discharge from the phagosome in order to retain the intracellular cycle as described above. As another level of control, LLO is activated by host factor,  $\gamma$ -interferon-inducible lysosomal thiol reductase (GILT), only within the vacuole. The lack of GILT leads to delayed escape of the pathogen from the vacuole (Singh, Jamieson et al. 2008). However, listeria can undergo a slow proliferative phase in macrophage vacuoles known as spacious Listeria-containing phagosomes (SLAPs). A low concentration of LLO at neutral pH inhibits phagosomal escape but renders LM replication sluggish in the non-degradative autophagic vacuole SLAPs-a crucial strategy to establish chronic infection (Birmingham, Canadien et al. 2008).

## 6. Tuberculosis

Tuberculosis is an air borne infectious disease. *Mycobacterium tuberculosis*, a highly, successful, gram-positive, acid-fast bacterium is the causative agent of the disease. It is still a leading cause of death in developing countries, claimed 1.7 million lives globally in 2009 (WHO 2010) (Figure 7). The bacterium has evolved with the host and discontinued drug regimens, poor patient management and diagnosis has led to the emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains. Furthermore, infection increases susceptibility to HIV that worsens the scenario by contributing to mortality and is difficult to treat especially in African continent. The bacterium is armored with a cell wall composed of glycolipids, fatty acids and other constituents. The slow growing nature of the bacterium ensures its persistence and the establishment of chronic infection, which requires long-term treatment with drugs (Kaufmann 2001).



**Figure 7. Global distribution of Tuberculosis.**

Map showing total number of tuberculosis reported cases per 100, 000 populations in each country of the world, according to the world health organization (WHO) 2010 report on TB.

Over the centuries of interaction with the host, this pathogen has developed very complex relation to an extent that it resides and multiplies within macrophages, a success achieved by conquering the killer cells itself –macrophages, the major cell population responsible for the clearance of pathogens.

### **6.1 Mouse model of tuberculosis**

As in human, aerosolized tuberculosis infection in mice is primarily a disease of lungs. However, bacilli burden differ between resistant C57BL/6 and susceptible BALB/c mice (Medina and North 1998; North and Medina 1998), indicating C57BL/6 mice control replication of tuberculosis. Inhalation of 100 mycobacterium leads to linear log phase increase in growth up to 10,000 folds by 3 weeks, followed by stationary phase to control infection, reflecting commencement of acquired immunity till mice succumb to infection after 8 months (Schell, Ealey et al. 1974). In contrast, susceptible strains do not stop *Mtb* growth following 3 weeks of linear log phase (Medina and North 1996; Medina and North 1999) rendering early death by uncontrolled bacilli burden. The inhaled mycobacteria ingested by alveolar macrophage, which can also disseminate in deeper tissues (Leemans, Juffermans et al. 2001) and eventually lyse to release bacteria that leads to generate cell-mediated immune response. Infected alveolar macrophages produce chemo-attractants like MCP-1 and RANTES and pro-inflammatory cytokines like TNF- $\alpha$  and IL-1 via activation of a transcription factor NF-kB, which in turns leads to further infiltration of monocytes to site of infection to form hypoxic aggregates of cells known as granuloma (Flynn and Chan 2001). In granulomatous environment activated antigen-presenting cells (APCs) delivers antigen to the periphery of granuloma consists mainly of activated T cells to generated adaptive immunity (Cosma, Sherman et al. 2003). CD4 T cells play important role in activating macrophages by producing IFN- $\gamma$ , which in turn produce IL-12 to induce Th1 response. IFN- $\gamma$  and TNF- $\alpha$  synergistically activate macrophages to produce reactive oxygen or nitrogen intermediates (ROI/RNI), which have strong anti-microbial properties. Activated macrophages inhibit growth of *Mtb* via production of reactive RNI (Chan, Xing et al. 1992; Chan, Tanaka et al. 1995). Thus, macrophages suppress



growth of phagocytosed bacilli and contain infection. However, role of nitric oxide in human remains subject of controversy.

Mycobacteria escape its destruction by hijacking many host factors, one of which targets the most decorated cells in terms of wide range of surface receptors, professional phagocyte -macrophages, which guaranteed the uptake of pathogen. The receptor-ligand interactions were enhanced in presence of cholesterol, which has been implicated to act as docking site for pathogens on macrophages and neutrophils (Gatfield and Pieters 2000; Peyron, Bordier et al. 2000). Once, internalized mycobacterium particularly arrest phagosomal degradative pathway at early stage, which prevents fusion with lysosomes and subsequent acidification by modulating influx of protons into phagosomes via ATPase (Armstrong and Hart 1975; Sturgill-Koszycki, Schlesinger et al. 1994; Ferrari, Langen et al. 1999). In addition, to that mycobacteria also inhibit apoptosis and macrophage bactericidal functions (Cosma, Sherman et al. 2003). This enables pathogen to evade microbicidal activity of macrophages thereby creating a hostile niche in early endosomes of macrophages. At this stage mycobacteria harness iron from the host via its iron scavenging molecules for its intracellular survival under nutrient and oxygen deprived condition in phagosomes and undergo non-replicating latent phase (Lounis, Truffot-Pernot et al. 2001). The transition of mycobacteria from dormant latent phase to active replicating phase requires catabolism of lipids, as later are rich source of energy during dormancy and present in ample quantity in granulomas (McKinney, Honer zu Bentrup et al. 2000).

## **6.2 Infection of macrophages with *Mycobacterium tuberculosis in vitro***

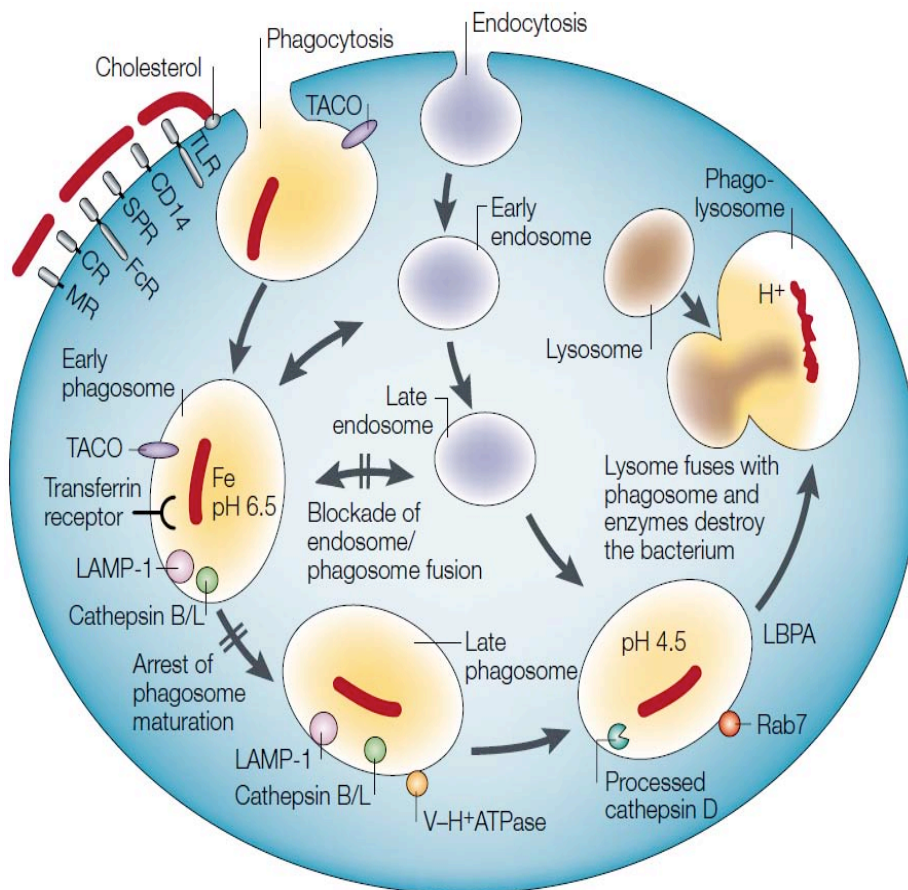
Macrophages possess antimicrobial functions during in vitro conditions (Suter 1952). Mycobacterium invades macrophages via variety of cell surface receptors. For example, complement receptors (CRs), Fcγ receptors and mannose receptors (Ernst 1998; Cambi, Koopman et al. 2005). The ability of mycobacteria to interact with diverse receptors attributed to its multifaceted cell wall (Brennan and Nikaido 1995). Receptor-specific entry determines the fate of mycobacteria once phagocytosed. Mycobacterial entry via CR3 results in

inhibition of macrophage activation, a key process to control infection and generate adaptive immune response. In contrast, entry via Fc receptors leads to respiratory burst by recruiting NADPH to phagosomes and an inflammatory response (Caron and Hall 1998). Mycobacterium also interact with either TLR2 or TLR4 in ligand specific manner, which initiate signaling cascades via Myd88 to activate NF- $\kappa$ B that induces pro-inflammatory cytokines (TNF- $\alpha$ , IL-6 and IL-1) in response to infection (Baeuerle and Henkel 1994; Medzhitov, Preston-Hurlburt et al. 1997). Differential role of TLR4 and TLR2 are reported in macrophages and in mice when challenged with non-pathogenic *Mycobacterium bovis* (BCG), suggesting their variable role in innate immunity (Heldwein, Liang et al. 2003). TNF- $\alpha$  together with IFN- $\gamma$  activate macrophages to exhibits its microbicidal activities, due to induction of NOS2 which produce nitric oxide (Walker and Lowrie 1981).

### 6.3 Phagolysosomes, an ideal niche for *Mtb*

The fusion of the most acidic compartments of the cells- lysosomes, with phagosomes is finely regulated process, which deliver hydrolytic enzymes in phagosome/endosomes that can degrade larger particles including phagocytosed microbes via acidic hydrolases. Previous studies, reported that this process could degrade microbes within 2 hours of phagocytosis (Cohn 1963) and a crucial event for antimicrobial properties of macrophages. Phagolysosomal pathway also interacts with other endocytic pathways, which involves GTPase of Ras family. The absence of Rab7 on mycobacterial phagosomes thought to be point of phagolysosomal arrest, revealed by Rab5 and EEA1 (early endosomal antigen 1) signature on early phagosomes and lack of Rab7 recruitment on late phagosomes (Desjardins, Huber et al. 1994; Via, Deretic et al. 1997). However, mycobacterial phagosomes acquire a late endosomal marker, LAMP1 (Lysosome-associated membrane protein 1) with Cathepsin D, an acid hydrolases (Clemens and Horwitz 1995). Furthermore, recruitment of tryptophan aspartate-containing coat (TACO) on viable mycobacterial phagosome results in inhibition of fusion with lysosomes as non-viable mycobacterial could not retain TACO (Figure 8). Interestingly, liver is relatively resistant to *Mtb* infection, a feature could be attributed to TACO, which is not

expressed in Kuffper cells, a resident macrophage population of liver (Ferrari, Langen et al. 1999). On the other hand, macrophages set up one of its dominant chemical armament, the hydrogen ion ( $H^+$ ) to kill invading pathogens. To counteract host immunity, *Mtb* block acidification of phagolysosomes via inhibiting proton pumps ATPases, which transfer  $H^+$  ions in vacuoles (Sturgill-Koszycki, Schlesinger et al. 1994). Recent, study shown that mycobacterium can survive in acidic compartments of activated macrophages by maintaining its intra-bacterial periplasmic pH to avoid influx of proton via acid resistant Rv3671c locus and remain in phagosomal niche in undefined mechanism (Vandal, Pierini et al. 2008) and *Mtb* also induces significant amount of ammonia in culture (Gordon, Hart et al. 1980), which further provide alkaline environment. Thus, *Mtb* inhibit acidification of phagosomes to survive in hostile environment within macrophages.



**Figure 8. The intracellular life of *Mycobacterium tuberculosis*.**

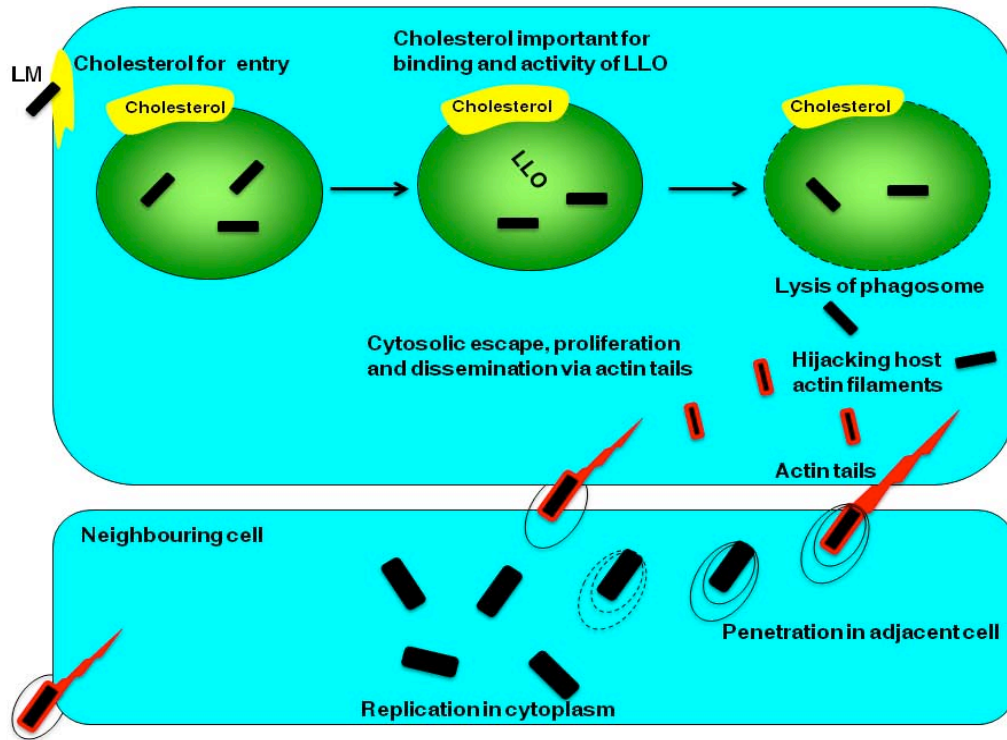
Both the endosomal and phagosomal pathways undergo interconnected maturation processes that merges at a late stage, prior to fusion with lysosomes. Cholesterol serves as a docking site that facilitates interactions between mycobacteria and surface receptors. The early phagosome-harbouring mycobacteria characteristically retain TACO, which apparently prevents its further maturation. *M. tuberculosis* inhibits phagosomal acidification (which occurs by means of a V-H<sup>+</sup>ATPase) and prevents fusion with the endosomal pathway. The arrest of phagosomal maturation is, however, incomplete and some phagosomes mature to form phagolysosomes. Although phagosome and endosome maturation form a continuum, distinct steps can be distinguished by means of different markers and tracers, some of which are shown. CR, complement receptor; FcR, receptor for the constant fragment of immunoglobulin; LAMP-1, lysosomal-associated membrane protein 1; LBPA, lysobiphosphatic acid; MR, mannose receptor; Rab7, member of the small GTPase family; SPR, surfactant protein receptor; TACO, tryptophane, aspartate-containing coat protein; TLR, Toll-like receptor; V-H<sup>+</sup>ATPase, vacuolar ATP-dependent proton pump. (Adapted from Kaufmann, S.H.E, 2001, *Nature Rev. Immunol.* Oct. 1:20).

## 7. Research covered in present study

Bacterial pathogens over the decades of interaction have evolved mechanisms to evade host microbicidal activities. Though, exit strategies revolve around central key processes yet they differ in their way of immune evasion by varying cellular and molecular mechanisms. The recent evidence indicates that statin has a beneficial effect on the outcome of infectious diseases. Here, we investigated the role of statins in mouse model of listeriosis and tuberculosis caused by intracellular pathogens *Listeria monocytogenes* and *Mycobacterium tuberculosis* respectively. We demonstrated a possible mechanism underlying the anti-microbial activity of statins in isolated macrophages. Furthermore, we investigated the advantages of statin therapy in peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM) from patients with Familial Hypercholesterolemia (FH) on outcome of *M.tb* infection.

### 7.1 Role of cholesterol in listeriosis

Role of cholesterol has been reported in uptake of *L. monocytogenes* in macrophages and other nonprofessional phagocytes (Figure 9). Listeriolysin O (LLO) is a cholesterol-dependent family of cytolysins, which inserts into the vacuolar membrane by binding cholesterol and forms pores that results in the phagosomal membrane disruption (Mengaud, Chenevert et al. 1987). Bacterial uptake of *L. monocytogenes* both in fibroblast and epithelial cell lines following M $\beta$ CD treatment was significantly reduced (Seveau, Bierne et al. 2004). Moreover, listerial entry via InB/Met signalling pathway is due to cholesterol-dependent activation of Rac1 and PI 3-Kinase within the domains of the membrane (Seveau, Tham et al. 2007). However, reported studies used cyclodextrins to deplete membrane cholesterol, which is potent cholesterol sequestrant and distort the membrane receptors crucial for entry into cells.

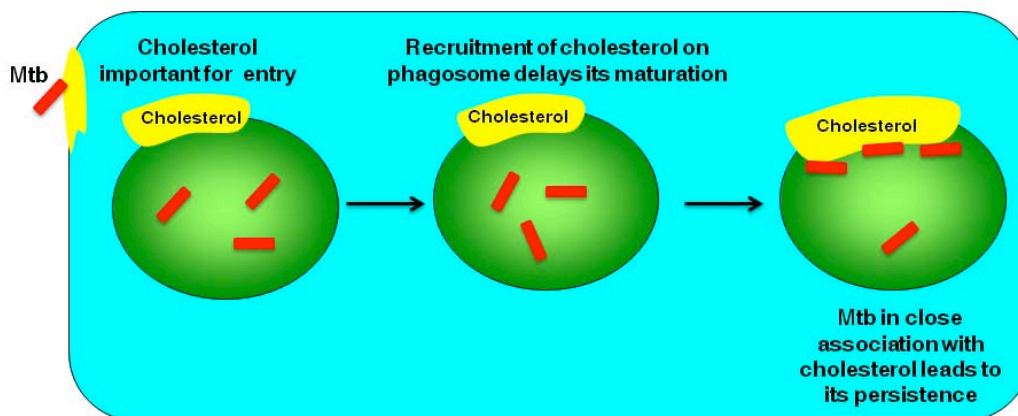


**Figure 9. Role of cholesterol in macrophages infected with *Listeria monocytogenes*.** Activity of cholesterol-dependent cytolysin, Listeriolysin O results in phagosomal escape, cytosolic proliferation and hijacking host actin filaments for cell-to-cell transmission.

## 7.2 Role of cholesterol in tuberculosis

Cholesterol has been implicated to be one of the crucial factors for pathogenesis of mycobacteria from uptake to persistence in macrophages. The uptake of *Mycobacterial spp.* in macrophages and neutrophils is cholesterol dependent (Gatfield and Pieters 2000; Peyron, Bordier et al. 2000) (Figure 10). Cholesterol esters reported to be accumulated in *Mtb* infected macrophages and pathogen able to retain the levels as compared to control (Kondo and Kanai 1976). Coating of *Mtb* with lipid complexes leads to decreased killing by oxidative burst and results in increased susceptibility in mice (Kondo and Kanai 1976). Role of cholesterol in imparting susceptibility to *Mtb* infection was established in mouse model of hypercholesterolemia (ApoE<sup>-/-</sup>), where bacilli burden was increased by 2-log in lungs (Martens, Arikan et al. 2008). Cholesterol is foremost component of lipid droplets, accumulation of later turns macrophages into foamy appearance, results in persistence of *Mtb* by providing lipid-rich nutrients in hypoxic human granulomas (Peyron, Vaubourgeix et al. 2008; Russell, Cardona

et al. 2009). Furthermore, cholesterol is recruited at the mycobacterial phagosomes following internalization and required for inhibition of lysosome with phagosomes (Jayachandran, Sundaramurthy et al. 2007). Fluvastatin increases Th1 cytokines in human PBMC infected with heat-killed avirulent strain (H37Ra) of *Mycobacterium tuberculosis* a immunoprotective response in caspase-1 dependent manner (Montero, Hernandez et al. 2000).



**Figure 10. Role of cholesterol in pathogenesis and persistence of *Mtb*.** Accumulation of cholesterol on phagosomal membrane delayed its maturation and ultimate fusion with degradative compartment lysosomes. Further macrophages harbor *Mtb* in close association with lipid bodies (rich source of cholesterol) result in persistence.

## 8. Observational studies of statins in humans

Statin study in human subjects revealed intriguing outcomes. Statin user veterans in US are less susceptible to tuberculosis as compared to non-statin users (Cirillo 2008). Recently, a meta-analysis of sixteen cohorts on statin therapy revealed the mortality benefits in patients of various bacterial infections (Tleyjeh, Kashour et al. 2009). Statin use has a beneficial effect on mortality in patients of bacteremia (Liappis, Kan et al. 2001; Thomsen, Hundborg et al. 2006).



## 9. Rationale for the proposed study

Cholesterol and its derivatives have been reported to augment inflammatory response (Zhu, Lee et al. 2008; Yvan-Charvet, Wang et al. 2010). On contrary, dynamics of cholesterol trafficking, accumulation and efflux have been reported to modulate by innate immune signaling (Maitra, Parks et al. 2009; Haas and Mooradian 2010). In this perspective, toll like receptors (TLR) have been implicated to engage with liver X receptors (LXR), a crucial lipid regulator following bacterial or viral infections. Further, reflecting role of innate immune signaling in modulating lipid metabolism (Castrillo, Joseph et al. 2003). It has been documented that host cholesterol is utilized by mycobacteria for its persistence (Pandey and Sasseti 2008).

However, major limitation of previous studies is the rapid sequestration of cholesterol by cyclodextrins, which promotes conformational changes in membrane and alters membrane receptors. This is crucial for the topological signaling of transmembrane receptors (Kabouridis, Janzen et al. 2000; Chen and Resh 2002) and such alterations in membrane could potentially inhibit uptake and internalization of pathogens both in professional and non-professional phagocytes.

Here, we used well-tolerated cholesterol lowering drugs, statins to modulate host de novo cholesterol biosynthesis pathway. Besides lowering cholesterol, statins has been implicated in many pleiotropic effects including anti-inflammatory (Liao and Laufs 2005) and immunomodulatory (Palinski 2000) functions.



## 10. Hypothesis

We hypothesized whether modulation of cholesterol biosynthesis pathway using statin may affect intracellular growth of intracellular pathogens during mouse model of listeriosis and tuberculosis.

## 11. Objectives

(A) To determine the effect of statin-mediated modulation of host cholesterol biosynthesis pathway during *Listeria monocytogenes* infection in mice and in isolated macrophages.

(B) To quantify intracellular growth of *Mycobacterium tuberculosis* following statin treatment in bone marrow-derived macrophages, mice and in mononuclear cells (PBMC) of patients with familial hypercholesterolemia (FH).

(C) To define the underlying mechanisms behind the anti-microbial activity of statins and immunomodulatory effects during tuberculosis.

## 12. References

- (World Market 2011). Statins: World market outlook 2011-2021. World Market 2011 Visiongain.
- Aderem, A. and R. J. Ulevitch (2000). "Toll-like receptors in the induction of the innate immune response." Nature **406**(6797): 782-787.
- Akira, S. and K. Takeda (2004). "Toll-like receptor signalling." Nat Rev Immunol **4**(7): 499-511.
- Allan, S. E., R. Broady, et al. (2008). "CD4+ T-regulatory cells: toward therapy for human diseases." Immunol Rev **223**: 391-421.
- Ando, H., T. Takamura, et al. (2000). "Cerivastatin improves survival of mice with lipopolysaccharide-induced sepsis." J Pharmacol Exp Ther **294**(3): 1043-1046.
- Argmann, C. A., C. G. Sawyez, et al. (2003). "Activation of peroxisome proliferator-activated receptor gamma and retinoid X receptor results in net depletion of cellular cholesteryl esters in macrophages exposed to oxidized lipoproteins." Arterioscler Thromb Vasc Biol **23**(3): 475-482.
- Armstrong, J. A. and P. D. Hart (1975). "Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival." J Exp Med **142**(1): 1-16.
- Ayukawa, Y., E. Yasukawa, et al. (2009). "Local application of statin promotes bone repair through the suppression of osteoclasts and the enhancement of osteoblasts at bone-healing sites in rats." Oral Surg Oral Med Oral Pathol Oral Radiol Endod **107**(3): 336-342.
- Ayyobi, A. F., S. H. McGladdery, et al. (2003). "Small, dense LDL and elevated apolipoprotein B are the common characteristics for the three major lipid phenotypes of familial combined hyperlipidemia." Arterioscler Thromb Vasc Biol **23**(7): 1289-1294.
- Baeuerle, P. A. and T. Henkel (1994). "Function and activation of NF-kappa B in the immune system." Annu Rev Immunol **12**: 141-179.
- Benati, D., M. Ferro, et al. (2010). "Opposite effects of simvastatin on the bactericidal and inflammatory response of macrophages to opsonized *S. aureus*." J Leukoc Biol **87**(3): 433-442.
- Birmingham, C. L., V. Canadien, et al. (2008). "Listeriolysin O allows *Listeria monocytogenes* replication in macrophage vacuoles." Nature **451**(7176): 350-354.
- Blanc, M., W. Y. Hsieh, et al. (2011). "Host Defense against Viral Infection Involves Interferon Mediated Down-Regulation of Sterol Biosynthesis." PLoS Biol **9**(3): e1000598.
- Blank, N., M. Schiller, et al. (2007). "Atorvastatin inhibits T cell activation through 3-hydroxy-3-methylglutaryl coenzyme A reductase without decreasing cholesterol synthesis." J Immunol **179**(6): 3613-3621.
- Bloch, K. (1965). "The biological synthesis of cholesterol." Science **150**(692): 19-28.
- Brennan, P. J. and H. Nikaido (1995). "The envelope of mycobacteria." Annu Rev Biochem **64**: 29-63.
- Brown, M. S. and J. L. Goldstein (1976). "Receptor-mediated control of cholesterol metabolism." Science **191**(4223): 150-154.

- Brown, M. S. and J. L. Goldstein (1996). "Heart attacks: gone with the century?" *Science* **272**(5262): 629.
- Brumeanu, T. D., R. Goldstein, et al. (2006). "Down-regulation of autoreactive T-cells by HMG CoA reductase inhibitors." *Clin Immunol* **119**(1): 1-12.
- Buhman, K. F., M. Accad, et al. (2000). "Mammalian acyl-CoA:cholesterol acyltransferases." *Biochim Biophys Acta* **1529**(1-3): 142-154.
- Cambi, A., M. Koopman, et al. (2005). "How C-type lectins detect pathogens." *Cell Microbiol* **7**(4): 481-488.
- Caron, E. and A. Hall (1998). "Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases." *Science* **282**(5394): 1717-1721.
- Castrillo, A., S. B. Joseph, et al. (2003). "Crosstalk between LXR and toll-like receptor signaling mediates bacterial and viral antagonism of cholesterol metabolism." *Mol Cell* **12**(4): 805-816.
- Catron, D. M., Y. Lange, et al. (2004). "Salmonella enterica serovar Typhimurium requires nonsterol precursors of the cholesterol biosynthetic pathway for intracellular proliferation." *Infection and immunity* **72**(2): 1036-1042.
- CDC (2011). "Multistate Outbreak of Listeriosis Associated with Cantaloupe, United States." *Morbidity and Mortality Weekly Report (MMWR)* **60**(39): 1357-1358.
- CFIA (2009). "Listeria monocytogenes outbreak." *Canadian Food Inspection Agency*.
- Chan, J., K. Tanaka, et al. (1995). "Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis." *Infect Immun* **63**(2): 736-740.
- Chan, J., Y. Xing, et al. (1992). "Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages." *J Exp Med* **175**(4): 1111-1122.
- Chang, T. Y., B. L. Li, et al. (2009). "Acyl-coenzyme A:cholesterol acyltransferases." *Am J Physiol Endocrinol Metab* **297**(1): E1-9.
- Chen, S. J., D. J. Rader, et al. (2000). "Prolonged correction of hyperlipidemia in mice with familial hypercholesterolemia using an adeno-associated viral vector expressing very-low-density lipoprotein receptor." *Mol Ther* **2**(3): 256-261.
- Chen, X. and M. D. Resh (2002). "Cholesterol depletion from the plasma membrane triggers ligand-independent activation of the epidermal growth factor receptor." *J Biol Chem* **277**(51): 49631-49637.
- Chinetti, G., J. C. Fruchart, et al. (2006). "Transcriptional regulation of macrophage cholesterol trafficking by PPARalpha and LXR." *Biochem Soc Trans* **34**(Pt 6): 1128-1131.
- Chinetti, G., S. Lestavel, et al. (2003). "Peroxisome proliferator-activated receptor alpha reduces cholesterol esterification in macrophages." *Circ Res* **92**(2): 212-217.
- Chomarat, P., J. Banchereau, et al. (2000). "IL-6 switches the differentiation of monocytes from dendritic cells to macrophages." *Nat Immunol* **1**(6): 510-514.
- Cirillo, D. J. (2008). "The effect of statin use on incident immune-mediated and infectious conditions among U.S. veterans." *PhD diss, University of Iowa*.

- Clemens, D. L. and M. A. Horwitz (1995). "Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited." The Journal of experimental medicine **181**(1): 257-270.
- Cohn, Z. A. (1963). "The fate of bacteria within phagocytic cells. I. The degradation of isotopically labeled bacteria by polymorphonuclear leucocytes and macrophages." J Exp Med **117**: 27-42.
- Conlan, J. W. and R. J. North (1994). "Neutrophils are essential for early anti-Listeria defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody." J Exp Med **179**(1): 259-268.
- Coppens, I., A. P. Sinai, et al. (2000). "Toxoplasma gondii exploits host low-density lipoprotein receptor-mediated endocytosis for cholesterol acquisition." The Journal of cell biology **149**(1): 167-180.
- Cosma, C. L., D. R. Sherman, et al. (2003). "The secret lives of the pathogenic mycobacteria." Annu Rev Microbiol **57**: 641-676.
- Cossart, P., M. F. Vicente, et al. (1989). "Listeriolysin O is essential for virulence of Listeria monocytogenes: direct evidence obtained by gene complementation." Infect Immun **57**(11): 3629-3636.
- Dabiri, G. A., J. M. Sanger, et al. (1990). "Listeria monocytogenes moves rapidly through the host-cell cytoplasm by inducing directional actin assembly." Proc Natl Acad Sci U S A **87**(16): 6068-6072.
- David L. McClure, Robert J Valuck, et al. (2007). "Systematic review and meta-analysis of clinically relevant adverse events from HMG CoA reductase inhibitor trials worldwide from 1982 to present." Pharmacoepidemiology and drug safety **16**: 132-143.
- de Chastellier, C. and P. Berche (1994). "Fate of Listeria monocytogenes in murine macrophages: evidence for simultaneous killing and survival of intracellular bacteria." Infect Immun **62**(2): 543-553.
- del Real, G., S. Jimenez-Baranda, et al. (2004). "Statins inhibit HIV-1 infection by down-regulating Rho activity." The Journal of experimental medicine **200**(4): 541-547.
- Desjardins, M., L. A. Huber, et al. (1994). "Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus." J Cell Biol **124**(5): 677-688.
- Dirks, A. J. and K. M. Jones (2006). "Statin-induced apoptosis and skeletal myopathy." Am J Physiol Cell Physiol **291**(6): C1208-1212.
- Drevets, D. A., P. J. Leenen, et al. (1993). "Complement receptor type 3 (CD11b/CD18) involvement is essential for killing of Listeria monocytogenes by mouse macrophages." J Immunol **151**(10): 5431-5439.
- Dumont, J., L. Cotoni (1921). "Bacille semblable a` celui du rouget du porc rencontre` dans le L.C.R. d'un me`ningitique." Ann. Inst. Pasteur **35**: 625-633.
- Dunne, D. W., D. Resnick, et al. (1994). "The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid." Proc Natl Acad Sci U S A **91**(5): 1863-1867.
- Eck, M. V., J. Oost, et al. (2005). "Role of the macrophage very-low-density lipoprotein receptor in atherosclerotic lesion development." Atherosclerosis **183**(2): 230-237.

- Ernst, J. D. (1998). "Macrophage receptors for *Mycobacterium tuberculosis*." *Infect Immun* **66**(4): 1277-1281.
- Evers, B. M., M. S. Farooqi, et al. (2010). "Hair growth defects in Insig-deficient mice caused by cholesterol precursor accumulation and reversed by simvastatin." *J Invest Dermatol* **130**(5): 1237-1248.
- Ferrari, G., H. Langen, et al. (1999). "A coat protein on phagosomes involved in the intracellular survival of mycobacteria." *Cell* **97**(4): 435-447.
- Flynn, J. L. and J. Chan (2001). "Immunology of tuberculosis." *Annu Rev Immunol* **19**: 93-129.
- Gafvels, M. E., L. G. Paavola, et al. (1994). "Cloning of a complementary deoxyribonucleic acid encoding the murine homolog of the very low density lipoprotein/apolipoprotein-E receptor: expression pattern and assignment of the gene to mouse chromosome 19." *Endocrinology* **135**(1): 387-394.
- Gatfield, J. and J. Pieters (2000). "Essential role for cholesterol in entry of mycobacteria into macrophages." *Science* **288**(5471): 1647-1650.
- Gillett, R. C., Jr. and A. Norrell (2011). "Considerations for safe use of statins: liver enzyme abnormalities and muscle toxicity." *Am Fam Physician* **83**(6): 711-716.
- Glynn, S. A., D. O'Sullivan, et al. (2008). "The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, simvastatin, lovastatin and mevastatin inhibit proliferation and invasion of melanoma cells." *BMC Cancer* **8**: 9.
- Goldstein, J. L., Hobbs, H.H, Brown, M.S (1995). "Familial Hypercholesterolemia: The metabolic and molecular bases of inherited diseases." *McGraw-Hill Book Co. New York 7th Edition*: 1981-2030.
- Gordon, A. H., P. D. Hart, et al. (1980). "Ammonia inhibits phagosome-lysosome fusion in macrophages." *Nature* **286**(5768): 79-80.
- Gordon, S. (2003). "Alternative activation of macrophages." *Nat Rev Immunol* **3**(1): 23-35.
- Goudriaan, J. R., P. J. Tacke, et al. (2001). "Protection from obesity in mice lacking the VLDL receptor." *Arterioscler Thromb Vasc Biol* **21**(9): 1488-1493.
- Haas, M. J. and A. D. Mooradian (2010). "Regulation of high-density lipoprotein by inflammatory cytokines: establishing links between immune dysfunction and cardiovascular disease." *Diabetes Metab Res Rev* **26**(2): 90-99.
- Harty, J. T. and M. J. Bevan (1995). "Specific immunity to *Listeria monocytogenes* in the absence of IFN gamma." *Immunity* **3**(1): 109-117.
- Heldwein, K. A., M. D. Liang, et al. (2003). "TLR2 and TLR4 serve distinct roles in the host immune response against *Mycobacterium bovis* BCG." *J Leukoc Biol* **74**(2): 277-286.
- Hiltunen, T. P., J. S. Luoma, et al. (1998). "Expression of LDL receptor, VLDL receptor, LDL receptor-related protein, and scavenger receptor in rabbit atherosclerotic lesions: marked induction of scavenger receptor and VLDL receptor expression during lesion development." *Circulation* **97**(11): 1079-1086.
- Holowka, D., J. A. Gosse, et al. (2005). "Lipid segregation and IgE receptor signaling: a decade of progress." *Biochim Biophys Acta* **1746**(3): 252-259.

- Ikonen, E. (2006). "Mechanisms for cellular cholesterol transport: defects and human disease." *Physiol Rev* **86**(4): 1237-1261.
- Jayachandran, R., V. Sundaramurthy, et al. (2007). "Survival of mycobacteria in macrophages is mediated by coronin 1-dependent activation of calcineurin." *Cell* **130**(1): 37-50.
- Jutras, I. and M. Desjardins (2005). "Phagocytosis: at the crossroads of innate and adaptive immunity." *Annu Rev Cell Dev Biol* **21**: 511-527.
- Kabouridis, P. S., J. Janzen, et al. (2000). "Cholesterol depletion disrupts lipid rafts and modulates the activity of multiple signaling pathways in T lymphocytes." *Eur J Immunol* **30**(3): 954-963.
- Kaesemeyer, W. H., R. B. Caldwell, et al. (1999). "Pravastatin sodium activates endothelial nitric oxide synthase independent of its cholesterol-lowering actions." *J Am Coll Cardiol* **33**(1): 234-241.
- Karumanchi, S. A. and R. Thadhani (2010). "Kidney complications: why don't statins always work?" *Nat Med* **16**(1): 38-40.
- Katano, H., L. Pesnicak, et al. (2004). "Simvastatin induces apoptosis of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines and delays development of EBV lymphomas." *Proc Natl Acad Sci U S A* **101**(14): 4960-4965.
- Kaufmann, S. H., G. A. Follows, et al. (1992). "Immunity to intracellular bacteria." *Mem Inst Oswaldo Cruz* **87 Suppl 5**: 91-94.
- Kaufmann, S. H. E. (2001). "How can immunology contribute to the control of tuberculosis?" *Nature Reviews Immunology* **1**(10): 20-30.
- Kelly, S. A., P. J. Goldschmidt-Clermont, et al. (1998). "Protein tyrosine phosphorylation mediates TNF-induced endothelial-neutrophil adhesion in vitro." *Am J Physiol* **274**(2 Pt 2): H513-519.
- Kobayashi, K., K. Oka, et al. (1996). "Reversal of hypercholesterolemia in low density lipoprotein receptor knockout mice by adenovirus-mediated gene transfer of the very low density lipoprotein receptor." *J Biol Chem* **271**(12): 6852-6860.
- Kocks, C., E. Gouin, et al. (1992). "L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein." *Cell* **68**(3): 521-531.
- Kolb-Maurer, A., S. Pilgrim, et al. (2001). "Antibodies against listerial protein 60 act as an opsonin for phagocytosis of *Listeria monocytogenes* by human dendritic cells." *Infect Immun* **69**(5): 3100-3109.
- Kondo, E. and K. Kanai (1976). "Accumulation of cholesterol esters in macrophages incubated with mycobacteria in vitro." *Jpn J Med Sci Biol* **29**(3): 123-137.
- Kondo, E. and K. Kanai (1976). "A suggested role of a host-parasite lipid complex in mycobacterial infection." *Jpn J Med Sci Biol* **29**(4): 199-210.
- Kozarsky, K. F., K. Jooss, et al. (1996). "Effective treatment of familial hypercholesterolaemia in the mouse model using adenovirus-mediated transfer of the VLDL receptor gene." *Nat Genet* **13**(1): 54-62.
- Kuby (2002). "Immunology." **5th Edition**.
- Kureishi, Y., Z. Luo, et al. (2000). "The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals." *Nat Med* **6**(9): 1004-1010.

- Lange, Y., M. H. Swaisgood, et al. (1989). "Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts." *J Biol Chem* **264**(7): 3786-3793.
- Lauer, S., J. VanWye, et al. (2000). "Vacuolar uptake of host components, and a role for cholesterol and sphingomyelin in malarial infection." *EMBO J* **19**(14): 3556-3564.
- Lawman, S., C. Mauri, et al. (2004). "Atorvastatin inhibits autoreactive B cell activation and delays lupus development in New Zealand black/white F1 mice." *J Immunol* **173**(12): 7641-7646.
- Leemans, J. C., N. P. Juffermans, et al. (2001). "Depletion of alveolar macrophages exerts protective effects in pulmonary tuberculosis in mice." *J Immunol* **166**(7): 4604-4611.
- Leenen, P. J., B. P. Canono, et al. (1994). "TNF-alpha and IFN-gamma stimulate a macrophage precursor cell line to kill *Listeria monocytogenes* in a nitric oxide-independent manner." *J Immunol* **153**(11): 5141-5147.
- Lemaitre, B., E. Nicolas, et al. (1996). "The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults." *Cell* **86**(6): 973-983.
- Leung, B. P., N. Sattar, et al. (2003). "A novel anti-inflammatory role for simvastatin in inflammatory arthritis." *J Immunol* **170**(3): 1524-1530.
- Li, Q., M. Tsujita, et al. (1997). "Selective down-regulation by protein kinase C inhibitors of apolipoprotein-mediated cellular cholesterol efflux in macrophages." *Biochemistry* **36**(40): 12045-12052.
- Liao, J. K. and U. Laufs (2005). "Pleiotropic effects of statins." *Annu Rev Pharmacol Toxicol* **45**: 89-118.
- Liappis, A. P., V. L. Kan, et al. (2001). "The effect of statins on mortality in patients with bacteremia." *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **33**(8): 1352-1357.
- Lounis, N., C. Truffot-Pernot, et al. (2001). "Iron and *Mycobacterium tuberculosis* infection." *J Clin Virol* **20**(3): 123-126.
- Mackanness, G. B. (1962). "Cellular resistance to infection." *J Exp Med* **116**: 381-406.
- Maitra, U., J. S. Parks, et al. (2009). "An innate immunity signaling process suppresses macrophage ABCA1 expression through IRAK-1-mediated downregulation of retinoic acid receptor alpha and NFATc2." *Mol Cell Biol* **29**(22): 5989-5997.
- Martens, G. W., M. C. Arian, et al. (2008). "Hypercholesterolemia impairs immunity to tuberculosis." *Infect Immun* **76**(8): 3464-3472.
- Matsuura, E., G. R. Hughes, et al. (2008). "Oxidation of LDL and its clinical implication." *Autoimmun Rev* **7**(7): 558-566.
- Maxfield, F. R. and I. Tabas (2005). "Role of cholesterol and lipid organization in disease." *Nature* **438**(7068): 612-621.
- Maxfield, F. R. and D. Wustner (2002). "Intracellular cholesterol transport." *J Clin Invest* **110**(7): 891-898.
- McKenney, J. M., M. H. Davidson, et al. (2006). "Final conclusions and recommendations of the National Lipid Association Statin Safety Assessment Task Force." *Am J Cardiol* **97**(8A): 89C-94C.

- McKinney, J. D., K. Honer zu Bentrup, et al. (2000). "Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase." *Nature* **406**(6797): 735-738.
- Medina, E. and R. J. North (1996). "Evidence inconsistent with a role for the Bcg gene (Nramp1) in resistance of mice to infection with virulent Mycobacterium tuberculosis." *J Exp Med* **183**(3): 1045-1051.
- Medina, E. and R. J. North (1998). "Resistance ranking of some common inbred mouse strains to Mycobacterium tuberculosis and relationship to major histocompatibility complex haplotype and Nramp1 genotype." *Immunology* **93**(2): 270-274.
- Medina, E. and R. J. North (1999). "Genetically susceptible mice remain proportionally more susceptible to tuberculosis after vaccination." *Immunology* **96**(1): 16-21.
- Medzhitov, R., P. Preston-Hurlburt, et al. (1997). "A human homologue of the Drosophila Toll protein signals activation of adaptive immunity." *Nature* **388**(6640): 394-397.
- Mengaud, J., J. Chenevert, et al. (1987). "Identification of the structural gene encoding the SH-activated hemolysin of Listeria monocytogenes: listeriolysin O is homologous to streptolysin O and pneumolysin." *Infect Immun* **55**(12): 3225-3227.
- Merx, M. W., E. A. Liehn, et al. (2004). "HMG-CoA reductase inhibitor simvastatin profoundly improves survival in a murine model of sepsis." *Circulation* **109**(21): 2560-2565.
- Montero, M. T., O. Hernandez, et al. (2000). "Hydroxymethylglutaryl-coenzyme A reductase inhibition stimulates caspase-1 activity and Th1-cytokine release in peripheral blood mononuclear cells." *Atherosclerosis* **153**(2): 303-313.
- Mukherjee, S. and F. R. Maxfield (2004). "Membrane domains." *Annu Rev Cell Dev Biol* **20**: 839-866.
- Mundy, G., R. Garrett, et al. (1999). "Stimulation of bone formation in vitro and in rodents by statins." *Science* **286**(5446): 1946-1949.
- Murray, P. J. and T. A. Wynn (2011). "Protective and pathogenic functions of macrophage subsets." *Nat Rev Immunol* **11**(11): 723-737.
- Neuhaus, O., S. Strasser-Fuchs, et al. (2002). "Statins as immunomodulators: comparison with interferon-beta 1b in MS." *Neurology* **59**(7): 990-997.
- North, R. J. and E. Medina (1998). "How important is Nramp1 in tuberculosis?" *Trends Microbiol* **6**(11): 441-443.
- Oka, K., K. W. Tzung, et al. (1994). "Human very-low-density lipoprotein receptor complementary DNA and deduced amino acid sequence and localization of its gene (VLDLR) to chromosome band 9p24 by fluorescence in situ hybridization." *Genomics* **20**(2): 298-300.
- Oskarsson, B. (2011). "Myopathy: five new things." *Neurology* **76**(7 Suppl 2): S14-19.
- Palinski, W. (2000). "Immunomodulation: a new role for statins?" *Nat Med* **6**(12): 1311-1312.
- Pamer, E. G. (2004). "Immune responses to Listeria monocytogenes." *Nat Rev Immunol* **4**(10): 812-823.



- Pandey, A. K. and C. M. Sasseti (2008). "Mycobacterial persistence requires the utilization of host cholesterol." *Proc Natl Acad Sci U S A* **105**(11): 4376-4380.
- Panichi, V., S. Paoletti, et al. (2006). "In vivo and in vitro effects of simvastatin on inflammatory markers in pre-dialysis patients." *Nephrol Dial Transplant* **21**(2): 337-344.
- Peyron, P., C. Bordier, et al. (2000). "Nonopsonic phagocytosis of *Mycobacterium kansasii* by human neutrophils depends on cholesterol and is mediated by CR3 associated with glycosylphosphatidylinositol-anchored proteins." *J Immunol* **165**(9): 5186-5191.
- Peyron, P., J. Vaubourgeix, et al. (2008). "Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for *M. tuberculosis* persistence." *PLoS Pathog* **4**(11): e1000204.
- Pfeffer, K., T. Matsuyama, et al. (1993). "Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection." *Cell* **73**(3): 457-467.
- Pucadyil, T. J., P. Tewary, et al. (2004). "Cholesterol is required for *Leishmania donovani* infection: implications in leishmaniasis." *Mol Biochem Parasitol* **133**(2): 145-152.
- Rader, D. J. (2006). "Molecular regulation of HDL metabolism and function: implications for novel therapies." *J Clin Invest* **116**(12): 3090-3100.
- Rodgers, W. and J. Zavzavadjian (2001). "Glycolipid-enriched membrane domains are assembled into membrane patches by associating with the actin cytoskeleton." *Exp Cell Res* **267**(2): 173-183.
- Rothe, J., W. Lesslauer, et al. (1993). "Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*." *Nature* **364**(6440): 798-802.
- Rothwell, C., A. Lebreton, et al. (2009). "Cholesterol biosynthesis modulation regulates dengue viral replication." *Virology* **389**(1-2): 8-19.
- Rub, A., R. Dey, et al. (2009). "Cholesterol depletion associated with *Leishmania* major infection alters macrophage CD40 signalosome composition and effector function." *Nat Immunol* **10**(3): 273-280.
- Russell, D. G., P. J. Cardona, et al. (2009). "Foamy macrophages and the progression of the human tuberculosis granuloma." *Nat Immunol* **10**(9): 943-948.
- Sadeghi, M. M., M. Collinge, et al. (2000). "Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein." *J Immunol* **165**(5): 2712-2718.
- Sakai, J., A. Hoshino, et al. (1994). "Structure, chromosome location, and expression of the human very low density lipoprotein receptor gene." *J Biol Chem* **269**(3): 2173-2182.
- Sakamoto, K., I. Wada, et al. (2011). "Inhibition of Rab1 GTPase and endoplasmic reticulum-to-Golgi trafficking underlies statin's toxicity in rat skeletal myofibers." *J Pharmacol Exp Ther*.
- Schell, R. F., W. F. Ealey, et al. (1974). "The influence of vaccination on the course of experimental airborne tuberculosis in mice." *J Reticuloendothel Soc* **16**(3): 131-138.

- Schmidt, H., R. Hennen, et al. (2006). "Association of statin therapy and increased survival in patients with multiple organ dysfunction syndrome." *Intensive Care Med* **32**(8): 1248-1251.
- Seftel, H. C., S. G. Baker, et al. (1980). "A host of hypercholesterolaemic homozygotes in South Africa." *Br Med J* **281**(6241): 633-636.
- Serbina, N. V., T. P. Salazar-Mather, et al. (2003). "TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection." *Immunity* **19**(1): 59-70.
- Seveau, S., H. Bierne, et al. (2004). "Role of lipid rafts in E-cadherin-- and HGF-R/Met--mediated entry of *Listeria monocytogenes* into host cells." *J Cell Biol* **166**(5): 743-753.
- Seveau, S., T. N. Tham, et al. (2007). "A FRET analysis to unravel the role of cholesterol in Rac1 and PI 3-kinase activation in the InlB/Met signalling pathway." *Cell Microbiol* **9**(3): 790-803.
- Shiloh, M. U., J. D. MacMicking, et al. (1999). "Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase." *Immunity* **10**(1): 29-38.
- Simons, K. and E. Ikonen (1997). "Functional rafts in cell membranes." *Nature* **387**(6633): 569-572.
- Simons, K. and W. L. Vaz (2004). "Model systems, lipid rafts, and cell membranes." *Annu Rev Biophys Biomol Struct* **33**: 269-295.
- Singh, R., A. Jamieson, et al. (2008). "GILT is a critical host factor for *Listeria monocytogenes* infection." *Nature* **455**(7217): 1244-1247.
- Soccio, R. E. and J. L. Breslow (2004). "Intracellular cholesterol transport." *Arterioscler Thromb Vasc Biol* **24**(7): 1150-1160.
- Strieter, R. M., S. L. Kunkel, et al. (1989). "Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-alpha, LPS, and IL-1 beta." *Science* **243**(4897): 1467-1469.
- Sturgill-Koszycki, S., P. H. Schlesinger, et al. (1994). "Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase." *Science* **263**(5147): 678-681.
- Suter, E. (1952). "The multiplication of tubercle bacilli within normal phagocytes in tissue culture." *J Exp Med* **96**(2): 137-150.
- Sutterwala, F. S., G. J. Noel, et al. (1998). "Reversal of proinflammatory responses by ligating the macrophage Fc gamma receptor type I." *J Exp Med* **188**(1): 217-222.
- Tabas, I. (2000). "Cholesterol and phospholipid metabolism in macrophages." *Biochim Biophys Acta* **1529**(1-3): 164-174.
- Takahashi, S., Y. Kawarabayasi, et al. (1992). "Rabbit very low density lipoprotein receptor: a low density lipoprotein receptor-like protein with distinct ligand specificity." *Proc Natl Acad Sci U S A* **89**(19): 9252-9256.
- Taylor, G. O. and E. O. Agbedana (1983). "A comparative study of plasma high-density lipoprotein cholesterol in two groups of Nigerians of different socio-economic status." *Afr J Med Med Sci* **12**(1): 23-28.
- Thomsen, R. W., H. H. Hundborg, et al. (2006). "Statin use and mortality within 180 days after bacteremia: a population-based cohort study." *Crit Care Med* **34**(4): 1080-1086.

- Tiebel, O., K. Oka, et al. (1999). "Mouse very low-density lipoprotein receptor (VLDLR): gene structure, tissue-specific expression and dietary and developmental regulation." *Atherosclerosis* **145**(2): 239-251.
- Tilney, L. G. and D. A. Portnoy (1989). "Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*." *J Cell Biol* **109**(4 Pt 1): 1597-1608.
- Tleyjeh, I. M., T. Kashour, et al. (2009). "Statins for the prevention and treatment of infections: a systematic review and meta-analysis." *Arch Intern Med* **169**(18): 1658-1667.
- Trinchieri, G. (1997). "Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-gamma)." *Curr Opin Immunol* **9**(1): 17-23.
- Unanue, E. R. (1997). "Studies in listeriosis show the strong symbiosis between the innate cellular system and the T-cell response." *Immunol Rev* **158**: 11-25.
- Unanue, E. R. (1997). "Why listeriosis? A perspective on cellular immunity to infection." *Immunol Rev* **158**: 5-9.
- Vainio, S. and E. Ikonen (2003). "Macrophage cholesterol transport: a critical player in foam cell formation." *Ann Med* **35**(3): 146-155.
- van Meer, G. (1989). "Lipid traffic in animal cells." *Annu Rev Cell Biol* **5**: 247-275.
- Vandal, O. H., L. M. Pierini, et al. (2008). "A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*." *Nat Med* **14**(8): 849-854.
- Varma, R. and S. Mayor (1998). "GPI-anchored proteins are organized in submicron domains at the cell surface." *Nature* **394**(6695): 798-801.
- Vazquez-Boland, J. A., C. Kocks, et al. (1992). "Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread." *Infect Immun* **60**(1): 219-230.
- Veerkamp, M. J., J. de Graaf, et al. (2002). "Diagnosis of familial combined hyperlipidemia based on lipid phenotype expression in 32 families: results of a 5-year follow-up study." *Arterioscler Thromb Vasc Biol* **22**(2): 274-282.
- Via, L. E., D. Deretic, et al. (1997). "Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7." *J Biol Chem* **272**(20): 13326-13331.
- Vilcek, J. (1998). "The cytokines: An overview." *The Cytokine Handbook*.
- Vogt, A., U. Kassner, et al. (2006). "Evaluation of the safety and tolerability of prolonged-release nicotinic acid in a usual care setting: the NAUTILUS study." *Curr Med Res Opin* **22**(2): 417-425.
- Walker, L. and D. B. Lowrie (1981). "Killing of *Mycobacterium microti* by immunologically activated macrophages." *Nature* **293**(5827): 69-71.
- Westcott, M. M., C. J. Henry, et al. (2010). "Dendritic cells inhibit the progression of *Listeria monocytogenes* intracellular infection by retaining bacteria in major histocompatibility complex class II-rich phagosomes and by limiting cytosolic growth." *Infect Immun* **78**(7): 2956-2965.
- WHO (2000). "Listeria in France: Disease Outbreak Report." *CCFH*.
- WHO (2010). "Global Tuberculosis Control." *WHO Report 2012*.

- Wierzbicki, A. S. (2001). "Lipid-lowering drugs in lupus: an unexplored therapeutic intervention." Lupus **10**(3): 233-236.
- Yagyu, H., E. P. Lutz, et al. (2002). "Very low density lipoprotein (VLDL) receptor-deficient mice have reduced lipoprotein lipase activity. Possible causes of hypertriglyceridemia and reduced body mass with VLDL receptor deficiency." J Biol Chem **277**(12): 10037-10043.
- Yvan-Charvet, L., N. Wang, et al. (2010). "Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses." Arteriosclerosis, thrombosis, and vascular biology **30**(2): 139-143.
- Zapolska-Downar, D., A. Siennicka, et al. (2004). "Butyrate inhibits cytokine-induced VCAM-1 and ICAM-1 expression in cultured endothelial cells: the role of NF-kappaB and PPARalpha." J Nutr Biochem **15**(4): 220-228.
- Zeiser, R., S. Youssef, et al. (2007). "Preemptive HMG-CoA reductase inhibition provides graft-versus-host disease protection by Th-2 polarization while sparing graft-versus-leukemia activity." Blood **110**(13): 4588-4598.
- Zhu, X., J. Y. Lee, et al. (2008). "Increased cellular free cholesterol in macrophage-specific Abca1 knock-out mice enhances pro-inflammatory response of macrophages." The Journal of biological chemistry **283**(34): 22930-22941.

# **Chapter 2**

## **Materials and Methods**

University of Cape Town

## Materials and methods

### 1. Mice

Mice were kept in individually ventilated cages under specific-pathogen-free conditions within the biomedical animal facility of the Health Science Faculty, University of Cape Town (UCT). All experiments were performed in accordance to guidelines approved by the Animal Ethics Research Board of UCT. Mice were aged (6-10 weeks) and sex matched for each experiment.

### 2. Ethics statement

#### Mice

This study was performed in strict accordance with the guidelines of the South African National Standard for the Care and Use of Animals for Scientific Purpose (SANS 10386:2008). All mouse experiments were performed according to protocols approved by the Animal Research Ethics Committee of the Health Sciences Faculty, University of Cape Town (Permit Number: 009/041). All surgery was performed under halothane anesthesia, and all efforts were made to minimize suffering of the animals.

#### Human

Patients with familial hypercholesterolemia were recruited by Lipid Clinic at Groote Schuur Hospital following written informed consent for their blood to be used in the study. All patient samples were strictly anonymized. The study was performed according to protocol approved by the Human Research Ethics Committee of the Health Sciences Faculty, University of Cape Town (Permit Number: 400/2009).

### 3. Bacterial cultures

#### 3.1 *Listeria monocytogenes* culture

*L. monocytogenes* (virulent EGD strain) and attenuated LM  $\Delta$ LLO was grown at 37°C in Tryptic-Soy Broth (Difco). LM (virulent EGD strain) and LM  $\Delta$ LLO strains was a gift from T. Chakraborty (Institute of Medical Microbiology, University of Giessen, Giessen, Germany). Mid-log phase cultures (O.D=0.3-0.6) were harvested, aliquoted and frozen at -80°C. After thawing and rigorous vortex, viable cell counts were determined by plating serial dilutions of cultures on

Tryptic-Soy agar plates followed by incubation at 37°C for 24 hours (Dai, Bartens et al. 1997).

### **3.2 *Mycobacterium tuberculosis* culture**

*M. tuberculosis* (virulent H<sub>37</sub>Rv) was grown at 37°C in Middlebrook 7H9 Broth (Difco) liquid medium supplemented with 10% Middlebrook OADC enrichment medium (BD Biosciences), 5% glycerol and 0.05% Tween 80. Mid-log phase cultures were harvested, aliquoted and frozen at -80°C. After thawing and rigorous vortexing, viable cell counts were determined by plating serial dilutions of cultures on Difco™ Middlebrook 7H10 Agar (BD Biosciences) plates followed by incubation at 37°C for 21 days (Guler, Parihar et al. 2011).

## **4. *In vitro* macrophage culture and infections**

### **4.1 Bone marrow-derived macrophages (BMDM)**

Bone marrow-derived macrophages (BMDM) were cultured as previously described (Holscher, Arendse et al. 2006). Briefly, bone marrow was harvested from the femurs of 8-10 weeks old C57BL/6 mice and cells were cultured for 10 days in Teflon coated plastic bags with Pluznik medium containing 10% fetal calf serum, 5% inactivated horse serum, DMEM, L929 conditioning medium, 100units/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 1% β-mercaptoethanol (all reagents from Gibco).

### **4.2 *Listeria monocytogenes* infection in BMDM**

Bone marrow-derived macrophages (5x10<sup>5</sup> cells/well) were cultured in presence of indicated concentrations of simvastatin (Sigma) or vehicle alone (final concentration 0.004% DMSO) overnight. Cells were then infected with *L. monocytogenes* (MOI=10) for 1 hour at 37°C in presence of simvastatin, followed by gentamicin (50 µg/ml) treatment for additional 1 hour to get rid of extracellular bacteria. Culture supernatant was collected at 12 hours post infection for cytokine detection by ELISA and NO<sub>2</sub><sup>-</sup> was detected by Griess reagent assay. At indicated time points macrophages were washed and lysed in 1ml of 0.1% Triton-x-100 and bacterial growth determined by 10-fold dilutions in PBS and plated in duplicates on tryptic soy agar plates. Bacterial colonies were counted after 24 hours of incubation at 37°C.

### 4.3 *Mycobacterium tuberculosis* infection in BMDM

BMDM ( $5 \times 10^5$  cells/well) were cultured overnight in presence of indicated concentrations of simvastatin or vehicle alone. Cells were then infected with *Mycobacterium tuberculosis* (MOI=5) for 4 hours at 37°C in presence of simvastatin, followed by gentamicin (100 µg/ml) treatment for additional 2 hours to remove extracellular bacteria. At indicated time points, cells were washed and lysed in 1ml of Triton-x-100 and viable bacilli were determined by 10-fold dilutions in PBS and plated in duplicates on Middlebrook 7H10 agar plates supplemented with 10% OADC enrichment and 0.5% glycerol. Bacterial colonies were counted after 21 days of incubation at 37°C.

### 4.4 Filipin staining in macrophages

BMDM ( $5 \times 10^5$  cells/well) were cultured overnight on coverslips either in presence of indicated concentration of simvastatin ± mevalonate (Sigma). Cells were then infected with *Listeria monocytogenes* or left non-infected. Filipin was reconstituted by injecting 1ml of DMSO under nitrogen fumes. Aliquotes were protected from light and stored in -20°C under nitrogen fumes. Cells were stained with filipin dye for 30 minutes in PBS (1:500) at room temperature (Bergy and Eble 1968). Coverslips were extensively washed and mounted for image acquisition on confocal microscope.

### 4.5 Cholesterol content in macrophages

Macrophages were treated as indicated above and sonicated on ice. Cell debris was then removed by centrifugation at 1500rpm for 5min at 4°C. Cholesterol content was measured in supernatant of total lysates of macrophages, using commercial enzyme-based cholesterol assay E2HL kit (EnzyChrome, BioAssay systems) (Rub, Dey et al. 2009).

### 4.6 Cytokine ELISAs

Bone marrow-derived macrophages ( $5 \times 10^5$  cells/well) were treated with simvastatin and infected with LM as indicated above. Culture supernatant was collected at 12 hours post infection and stored at -20C until ELISA was



performed. Briefly, sandwich ELISAs were performed to determine cytokine levels in cell supernatants. Maxisorp microtitre plates (Nunc, Reskilde, Denmark) were coated with 50µl purified anti-IL-12p40, anti-TNF- $\alpha$  and anti-IL-6 (BD Pharmingen) diluted in phosphate buffered saline (PBS, pH 9.5) and incubated overnight at 4°C. Plates were blocked for 3hrs at 37°C and serially diluted standards, purified recombinant IL-12p40, TNF- $\alpha$  or IL-6 (BD Pharmingen) were added. Cell supernatants were then added, diluted in ELISA dilution buffer and incubated overnight at 4°C. Biotinylated anti-mouse secondary antibodies for IL-12p40, TNF- $\alpha$  and IL-6 (BD Pharmingen) were added for 3h at 37°C followed by enzyme alkaline phosphatase (AP) labeled streptavidin. ELISA wash buffer was used to wash plates 4x between each step. Subsequently, plates were incubated with 4-nitrophenyl phosphate disodium salt hexahydrate (NPP) Substrate (Sigma), for 10 minutes and absorption measured at 405nm (Mohrs, Ledermann et al. 1999).

#### 4.7 Nitric oxide measurement

A nitric oxide standard ( $\text{Na}_2\text{NO}_3$ , 2mM) and samples were serially diluted in DMEM on 96-well plate. 25µl of Griess reagent A = Sulfanilamide (1% sulfanilamide in 3% phosphoric acid) was then added on the plate followed by equal volume of Griess reagent B = NED solution (N-1-naphthyl ethylenediamine dihydrochloride). Plate was gently rocked to mix and incubated for 5-10minutes till pink color develops across standards and then absorption was measured at 540nm (Nickdel, Roberts et al. 2001).

#### 4.8 FACS analysis for MHC-II expression

IFN- $\gamma$  (100 units/ml) activated and non-activated BMDM were treated with either simvastatin (100 µM) or simvastatin in combination with mevalonate (100 µM) and infected with LM as indicated above. At 1 hour post infection, cells were washed to remove extracellular bacteria and transferred into FACS tubes. Media was removed by centrifugation at 1200rpm for 5minutes at 4°C, the macrophage cell pellet was then stained with MHC-II antibody (1:320) in 50 µl FACS buffer containing 1% heat-inactivated rat serum to block the Fc region of the added antibodies and thus prevent non-specific binding with Fc receptors in

the sample. Antibody mix and cells were slightly vortexed and incubated on ice for 30 minutes before washing off unbound antibody in 500  $\mu$ l FACS buffer by spin down cells at 1200rpm for 5 minutes at 4°C. Finally, stained cells were resuspended in 300  $\mu$ l FACS buffer for acquisition. To preserve the integrity of antibodies and cells, all steps were performed in the dark.

#### 4.9 MTT assay for cell viability and cytotoxicity

Cell viability of BMDM ( $1 \times 10^5$ ) treated with statins or methyl- $\beta$ -cyclodextrin (M $\beta$ CD) for indicated duration was quantified by reduction of yellow tetrazolium salt MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) by mitochondrial enzymes of living cells. At 24, 48 and 72 hours time points; 10  $\mu$ l of MTT (5mg/ml) was added, followed by incubation at 37°C for 2 hours. Purple formazan was solubilised in 0.04N HCL in isopropanol, incubated on shaker for 30 min before reading absorbance at 570nm (Ferrari, Fornasiero et al. 1990).

#### 4.10 Phagocytosis assay

BMDM ( $5 \times 10^5$  cells/well) were treated overnight on coverslips in 24-well plates with simvastatin  $\pm$  mevalonate, methyl- $\beta$ -cyclodextrin  $\pm$  cholesterol or cytochalasin D (5 $\mu$ M). Cells were then incubated with FITC-labelled beads (4.5 $\mu$ m in diameter, Dynabeads, Invitrogen) at ratio of 10 beads to 1 macrophage (10:1). After 1 hour at 37°C, non-adherent beads were removed with ice cold PBS and cells were fixed in 4% paraformaldehyde and stained with rhodamine-phalloidin to stain actin filaments (Kerrigan, Dennehy et al. 2009). Four fields were photographed by LSM 510 confocal microscope. Quantification of internalized beads was based on 250 macrophages per treatment.

#### 4.11 Immunofluorescence

Immunofluorescence was performed as described elsewhere. Bone marrow-derived macrophages ( $5 \times 10^5$ ) were cultured on coverslips in 24-well plates or  $1 \times 10^5$  in 96-well plates overnight. Macrophages were treated in presence of simvastatin or vehicle control for 24 hours. Cells were infected with *M. tuberculosis* (MOI=5) for 2 hours at 37°C. Cells were then washed, fixed with paraformaldehyde (4%), quenched with ammonium chloride (50 mM) and

permeabilized with Triton-x-100 (0.2%). Cells were incubated in blocking buffer (3% BSA, 0.5% Tween-20) and incubated with the primary antibodies (Santa Cruz) diluted in blocking buffer against early and late endosomal markers goat anti-EEA1 (1:100), goat anti-LAMP1 (1:100), rabbit anti-LAMP3 (1:300) & rabbit anti-Cathepsin D (1:300) and autophagy marker LC3-II (1:300). Primary antibodies were visualized by staining with Alexa 546 conjugated with anti-goat or rabbit (1:1000) antibodies (Invitrogen). Coverslips were then mounted with either mowiol or vectashield with nuclear stain DAPI. Images were acquired and analyzed by confocal microscopy (Zeiss LSM 510).

#### 4.12 Quantification of fluorescent images

Images from confocal microscope were analyzed by LSM software or images were opened in separate color channels and quantified for colocalization between GFP-expressing pathogen (green) and phagosomal/autophagy markers (red) using MATLAB image analysis software, where linear pixel classifier was used to find the bacteria. Marker-controlled watershed was used to segment individual cells. The canny edge detector was used to find the cell markers. In 2D, the cell markers and pathogen were aligned to find co-localization. Four fields were acquired for each replicate of duplicates consisting total of 600 cells/sample (BMDM) and 200 cells/sample (PBMC).

### 5. *In vivo* infections

#### 5.1 Simvastatin treatment and *Listeria monocytogenes* infection in mice

Simvastatin was reconstituted in DMSO containing PBS or vehicle control administered intraperitoneally with 10 mg/kg/day and 20 mg/kg/day for 12 days. On day 11, mice were infected intraperitoneally with  $2 \times 10^5$  *Listeria monocytogenes*. Serial dilutions of inoculum were plated to determine the infection dose. On day 14 (3 days post infection), blood was collected followed by euthanasia to determined bacterial titers in liver and spleen.

#### 5.2 Serum cholesterol measurement in mice

Blood samples for serum cholesterol levels were drawn and collected in microtainer tubes via tail bleeding. Blood were allowed to clot at room temperature for 3 hours followed by centrifugation at 8000rpm for 12 minutes.

Serum which was separated on upper part of the gel in microtainer tube, harvested in eppendorf tube and stored in -20°C until cholesterol assay. The serum cholesterol was determined using enzyme-based assay kit KAT as per instructions. Briefly, cholesterol esters from the samples were catalyzed with cholesterol esterase, and then the free cholesterol was oxidized in the presence of cholesterol oxidase. The resulting hydrogen peroxide reacted with 4-aminoantipyrine and p-hydroxybenzoate-producing red quinoneimine. The intensity of the red color produced was directly proportional to the amount of cholesterol in the sample and was detected spectrophotometrically at a wavelength of 500 nm (Allain, Poon et al. 1974).

### **5.3 Preparation of tissue homogenates for enumeration of bacterial loads**

Spleens and livers were aseptically collected, weighed and homogenized in 0.1% Triton-x-100 from day 3 post-infected mice. Tissue homogenates were plated on tryptic soy agar and incubated for 24 hours to determine the bacterial burden per organ.

In case of *Mtb*, bacterial loads in lungs and spleen were determined at different time points post-infection. Briefly, organs from euthanized mice were removed aseptically, weighed and homogenized in 0.04% Tween 80. Ten-fold dilutions were plated in duplicate onto Middlebrook 7H10 agar plates supplemented with 10% OADC and 0.5% glycerol, and incubated at 37°C for 21 days before colonies were counted.

### **5.4 Simvastatin treatment and aerosol inhalation with *Mtb***

Mice were treated with simvastatin, atorvastatin and rosuvastatin (20 mg/kg q.o.d) or vehicle control for 6 weeks (from week -2 to +4). Stock solutions of *M. tuberculosis* were thawed and aspirated through a 29-gauge needle for 30 times to disrupt clumping. Pulmonary infection was performed using an inhalation exposure system (model A4224, Glas-Col). To infect mice with a natural route of infection with a dose of 400 CFU/lung, animals were exposed for 40 min to an aerosol generated by nebulising approximately 6 ml of a suspension containing  $2 \times 10^6$  live bacteria. The dose was determined in the lungs of one-day post-infected mice.

### 5.5 Monitoring of Mtb-infected mice

Body weight of Mtb-infected mice was measured weekly. If infected mice lost more than 20% of their original body weight or showed severe signs of illness, such as hunched up posture, coat staring, immobility and general lack of grooming, mice were considered moribund and were euthanized.

### 5.6 Histopathological examinations

Portions of spleen and liver (in case of LM) and lungs (in case of Mtb) were fixed in 4% formalin and embedded in paraffin. Serial sections of tissue specimens were obtained for routine hemotoxylin and eosin (H & E) staining for inflammatory cells (Herbert, Holscher et al. 2004).

## 6. Western blot analysis

### 6.1 Preparation of macrophage whole-cell lysates

For Western blot analysis, whole-cell lysates were prepared from macrophages ( $2 \times 10^6$ ) infected with Mtb (MOI=5) for 2 hours. Macrophages were either not treated or cultured in presence of simvastatin (100  $\mu$ M) for 48 hours in 6-well plates pre-infection. Cells were then washed with warm medium to remove extracellular bacteria and lysed in ice-cold RIPA buffer containing protease inhibitor at 250  $\mu$ l/well at 4°C for 1 hour. Total cell lysates were centrifuged at 14000 rpm for 10 minutes at 4°C to remove cellular debris. Supernatants were transferred to new Eppendorf tube and stored at -80°C (Sisk, Gourley et al. 2000).

### 6.2 Protein content and Western blot

Protein concentrations were determined using BCA assay (Thermo Fisher). Equal amount of protein samples (30ug-45ug) were electrophoresed on 12% SDS-PAGE gel. The gel was transferred to a nitrocellulose membrane (Sigma). The membrane was incubated with anti-EEA1, LAMP1 and LC3-II primary antibody (1:800) at 4°C overnight. Membrane was then washed extensively and incubated with HRP-conjugated secondary antibody (1:10,000) for 1 hour at room temperature. Immunoblots were developed using SuperSignal West Dura

substrate (Thermo Scientific) (Parikh, Childress et al. 2010). Images were captured using UVP system.

## **7. Human mononuclear cells (PBMC) and macrophages culture**

### **7.1 Isolation of PBMC and monocyte-derived macrophages (MDM)**

Human peripheral blood (16 ml) samples were withdrawn from patients with familial hypercholesterolemia (FH) following informed consent from the Lipid clinic, Groote Schuur Hospital (Cape Town, South Africa) in cell preparation tubes (BD, CPT). Similarly, equal volume of blood was withdrawn from healthy donors. PBMC were separated by centrifugation. Cells were washed extensively in PBS and seeded at  $2 \times 10^5$  cells/well on a 96-well plate in RPMI 1640/10% FCS for 4 hours at 37°C. After 4 hours of adherence cells were washed gently to remove non-adherent cells.  $2 \times 10^6$  mononuclear cells/well were seeded in 24-well plates in complete RPMI and cultured for 7 days to generate monocyte-derived macrophages (MDM) (Paul, Laochumroonvorapong et al. 1996).

### **7.2 Intracellular growth of *Mtb* in human PBMC and MDM**

Cells were incubated overnight in presence of simvastatin (100 µM) or medium alone. Cells were then infected with *Mtb* at MOI=5 (Mononuclear cells) and MOI=2 (MDM) for 4 hours. After 4 hours of infection extracellular bacteria were removed by three washes with warm medium. Monocytes and MDM were then cultured in complete RPMI medium supplemented with gentamicin (25 µg/ml) for 72 hours. After 72 hours of post-infection, cells were washed and lysed in 0.1% Triton-X-100 for 5 minutes. Cell lysates were then diluted in PBS and plated on 7H10 agar plates to determine growth of *Mtb*.

## **8. Statistical analysis**

All data were analyzed using either student *t*-test (two-tailed with unequal variance) or one-way-ANOVA, Dunnett's post-test. A 'p' value of less than 0.05 was considered significant.

## 9. References

- Allain, C. C., L. S. Poon, et al. (1974). "Enzymatic determination of total serum cholesterol." *Clin Chem* **20**(4): 470-475.
- Bergy, M. E. and T. E. Eble (1968). "The filipin complex." *Biochemistry* **7**(2): 653-659.
- Dai, W. J., W. Bartens, et al. (1997). "Impaired macrophage listericidal and cytokine activities are responsible for the rapid death of *Listeria monocytogenes*-infected IFN-gamma receptor-deficient mice." *J Immunol* **158**(11): 5297-5304.
- Ferrari, M., M. C. Fornasiero, et al. (1990). "MTT colorimetric assay for testing macrophage cytotoxic activity in vitro." *J Immunol Methods* **131**(2): 165-172.
- Guler, R., S. P. Parihar, et al. (2011). "Blocking IL-1alpha but not IL-1beta increases susceptibility to chronic *Mycobacterium tuberculosis* infection in mice." *Vaccine* **29**(6): 1339-1346.
- Herbert, D. R., C. Holscher, et al. (2004). "Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology." *Immunity* **20**(5): 623-635.
- Holscher, C., B. Arendse, et al. (2006). "Impairment of alternative macrophage activation delays cutaneous leishmaniasis in nonhealing BALB/c mice." *Journal of immunology* **176**(2): 1115-1121.
- Kerrigan, A. M., K. M. Dennehy, et al. (2009). "CLEC-2 is a phagocytic activation receptor expressed on murine peripheral blood neutrophils." *J Immunol* **182**(7): 4150-4157.
- Mohrs, M., B. Ledermann, et al. (1999). "Differences between IL-4- and IL-4 receptor alpha-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signaling." *J Immunol* **162**(12): 7302-7308.
- Nickdel, M. B., F. Roberts, et al. (2001). "Counter-protective role for interleukin-5 during acute *Toxoplasma gondii* infection." *Infect Immun* **69**(2): 1044-1052.
- Parikh, A., C. Childress, et al. (2010). "Statin-induced autophagy by inhibition of geranylgeranyl biosynthesis in prostate cancer PC3 cells." *Prostate* **70**(9): 971-981.
- Paul, S., P. Laochumroonvorapong, et al. (1996). "Comparable growth of virulent and avirulent *Mycobacterium tuberculosis* in human macrophages in vitro." *J Infect Dis* **174**(1): 105-112.
- Rub, A., R. Dey, et al. (2009). "Cholesterol depletion associated with *Leishmania major* infection alters macrophage CD40 signalosome composition and effector function." *Nat Immunol* **10**(3): 273-280.
- Sisk, T. J., T. Gourley, et al. (2000). "MHC class II transactivator inhibits IL-4 gene transcription by competing with NF-AT to bind the coactivator CREB binding protein (CBP)/p300." *J Immunol* **165**(5): 2511-2517.

# **Chapter 3**

## **Results**

University of Cape Town



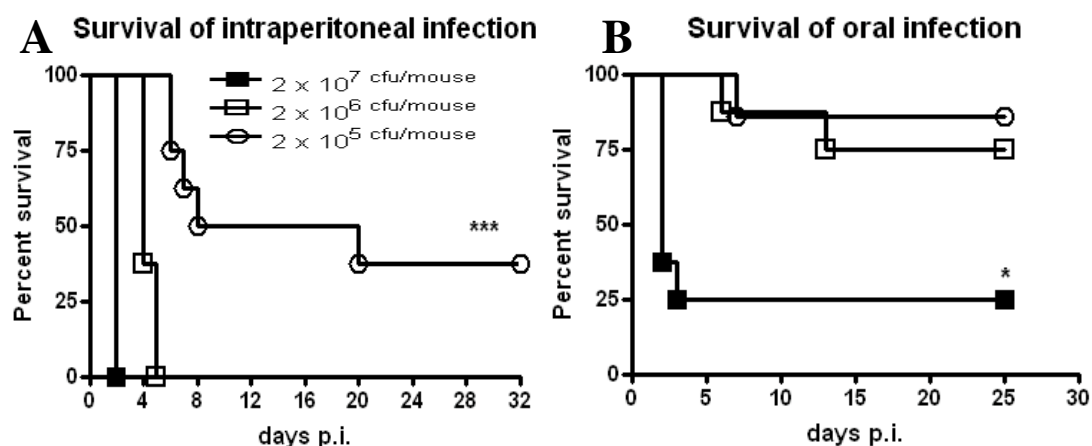
## Results

The aim the study was to investigate the role of statins during *Listeria monocytogenes* and *Mycobacterium tuberculosis* infection in mice. This chapter describes the results of the study in three sections. The first section describes dose optimization, route of infection and the selection of statins. The second section investigates the role of statins during *L. monocytogenes* infection in mice and the third section describes the role of statins during *M. tuberculosis* infection in mice, human macrophages and in patients with familial hypercholesterolemia (FH) on statin therapy.

### 1. Optimization

#### 1.1. Intraperitoneal versus oral route of *L. monocytogenes* infection

We compared the mortality of mice using two routes of infection of *L. monocytogenes* (LM), intraperitoneal (Figure 1A) or oral (Figure 1B). Different infectious doses were tested to determine the 50% lethal dose (LD<sub>50</sub>). Using  $2 \times 10^6$  LM bacteria, 75% of mice infected orally survived, whereas intraperitoneal infection of  $2 \times 10^6$  LM resulted in death of all mice. Moreover, 50% of mice infected intraperitoneally survived at the dose of  $2 \times 10^5$  LM bacteria as compared to nearly 90% survived on oral infection at the same dose. The route of LM administration also affected the outcome of infection. The intraperitoneal route resulted in a systemic infection with higher bacterial replication by escaping the first line of defense the intestinal barrier and subsequently gut-associated innate immune response. In contrast, the oral route caused a localized infection with lower bacterial replication due to reduced invasion into the wall of the intestine. Although the oral mode of LM infection better mimics the course of human disease, a limiting factor is that it requires a very high initial inoculum, as rodents lack E-cadherins that impair the invasion of LM (Gaillard, Berche et al. 1991). Therefore, subsequent studies with LM infection were performed using the intraperitoneal (i.p.) route of infection with  $2 \times 10^5$  LM.



**Figure 1. Impact of the route of *L. monocytogenes* infection on survival of mice.**

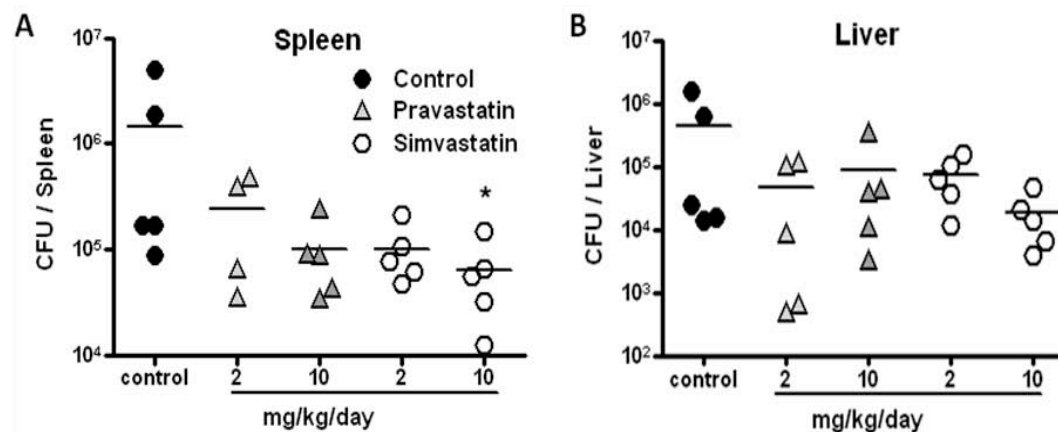
C57BL/6 mice were infected with *Listeria monocytogenes* either intraperitoneally (A) or orally (B). Mice groups were infected with three different inoculums  $2 \times 10^7$ ,  $2 \times 10^6$  and  $2 \times 10^5$  CFU/mouse. Results are from one experiment consists of 8 mice/group and analyzed by Kaplan-Meier survival curves and log rank test \*  $P < 0.05$  versus  $2 \times 10^6$  LM, \*\*\*  $P < 0.001$  versus  $2 \times 10^7$  LM.

## 1.2 Selection and dose optimization of statins in mice

The physico-chemical properties of statins play important roles in their diffusion through cell membranes. Statins are categorized in two groups according to their solubility in water (hydrophilic) or lipids (lipophilic). The difference in solubility is reflected in their ability to nonselectively cross cell membranes by passive diffusion. Hydrophilic statins such as pravastatin is administered as the active open hydroxy-acid form, which does not easily cross cell membranes and is absorbed mainly via selective membrane carriers of hepatocytes. On the other hand, lipophilic statins such as simvastatin, atorvastatin and rosuvastatin are administered as inactive lactones and are taken up by hepatocytes mainly through passive diffusion that in turn are converted into the active hydroxyl-acid form in order to inhibit the HMG-CoA reductase (Hamelin and Turgeon 1998). Here, we tested pravastatin (hydrophilic) and simvastatin (lipophilic) to study the effect of different statins in inducing protective host responses against bacterial pathogens.

Statins were administered at doses 2 or 10 mg/kg/day in order to establish the host protective effects in mice to *L. monocytogenes* infection. In mice, statins are administered at higher doses compared to humans (0.1-1 mg/day bodyweight) because lower doses would highly up-regulate the activity of the target enzyme HMGCo-A reductase (Kita, Brown et al. 1980). In the present study, we

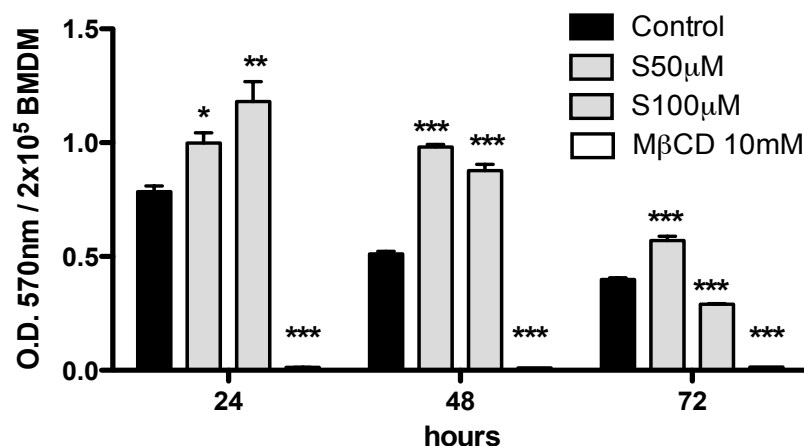
compared the bacterial burden between mice treated with 2 or 10 mg/kg/day of statins (Figure 2). Doses of 10 mg/kg/day significantly reduced the bacterial burden in both spleen and liver. Therefore, subsequent infection studies with statins were performed with 10 mg/kg/day.



**Figure 2. Effect of different doses and types of statins on *L. monocytogenes* burden in mice.** C57BL/6 female mice were treated with simvastatin and pravastatin. Mice were administered with two doses of each statin (2 mg/kg/day & 10 mg/kg/day) or the control by intraperitoneal injection for 9 days. The mice were infected intraperitoneally with  $2 \times 10^5$  *Listeria monocytogenes* on day 6 and then sacrificed on day 9. Bacterial loads were determined in spleen (A) and liver (B) homogenates at 3 days post-infection. Results are shown as mean  $\pm$  SEM from one experiment which consisted of  $n=5$  mice/group, \*  $P < 0.05$  versus control.

### 1.3 Effect of simvastatin on macrophage viability and proliferation

Statins were reported to inhibit cell proliferation (Denoyelle, Albanese et al. 2003; Glynn, O'Sullivan et al. 2008). However, such studies were performed on cancer cell lines, which have less cellular efficacy of statins. Here, we used the lactone form of simvastatin, and showed that the lower dose increased cell proliferation up to 72 hours compared to control. However, increasing the concentration resulted in inhibition of cell proliferation after 72 hours of incubation. In addition, we used methyl- $\beta$ -cyclodextrin (M $\beta$ CD) to deplete membrane cholesterol. Strikingly, M $\beta$ CD treatment reduced macrophage viability and proliferation already at 24 hours post treatment (Figure 3). Taken together these findings suggest that simvastatin was found to have no cytotoxicity effect after 48 hours at concentrations of 50-100  $\mu$ M. Therefore, 100  $\mu$ M of statins were used for subsequent in vitro infection studies.

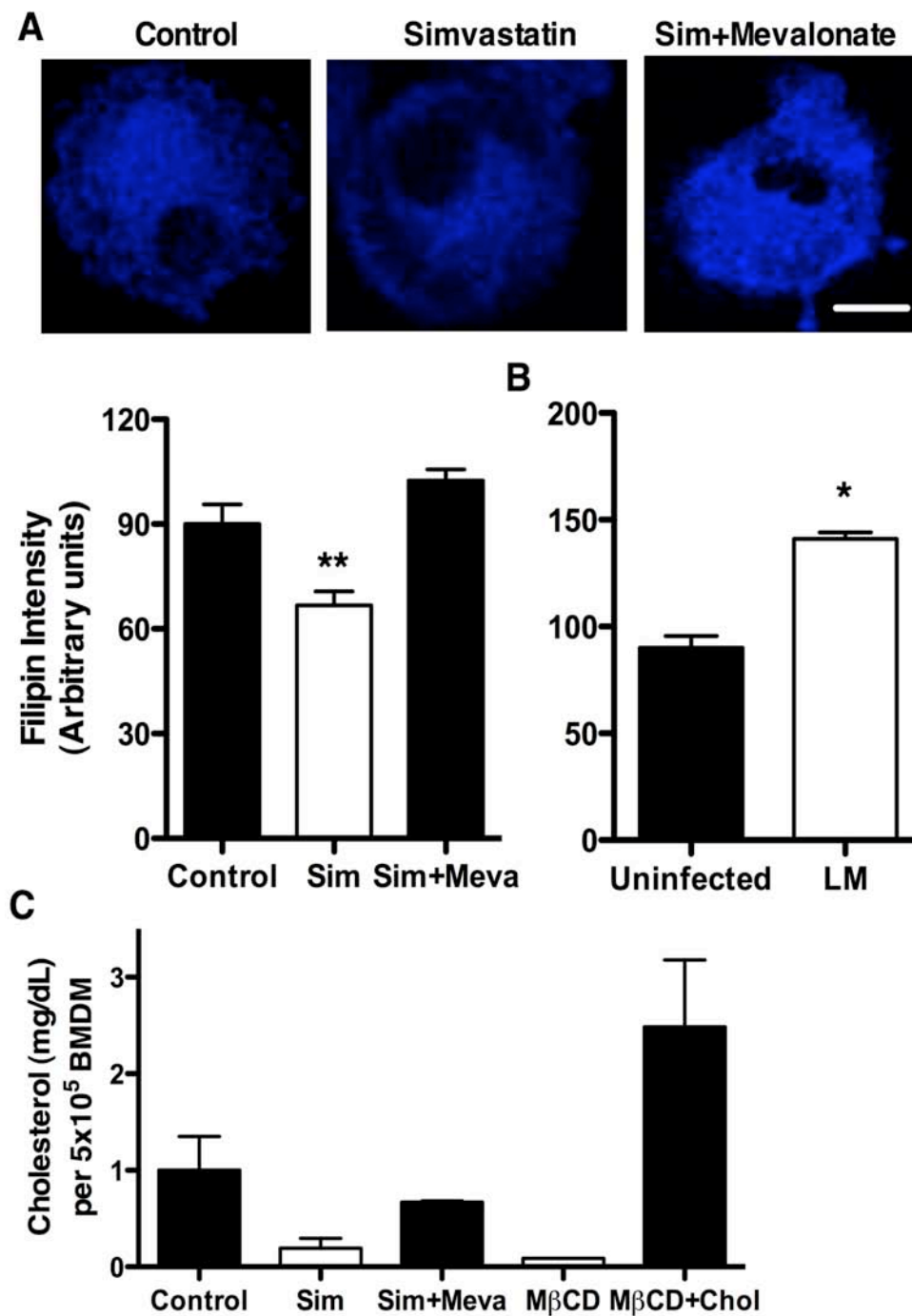


**Figure 3. Effect of cholesterol depletion on cell viability and proliferation.**

Cell viability was measured at indicated time points in the presence of simvastatin (S) and methyl- $\beta$ -cyclodextrin (M $\beta$ CD) or control (0.04% DMSO) by enzymatic MTT assay. All data are shown as mean  $\pm$  SEM of O.D. at 570nm from triplicate cultures one-way ANOVA followed by Dunnett's post-test and representative of two independent experiments, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  compared to control.

#### 1.4 Simvastatin decreased intracellular cholesterol levels in macrophages

To test whether simvastatin is able to decrease host cholesterol biosynthesis during LM infection, macrophages were stained with filipin, a fluorescent dye that specifically binds to cholesterol esters (Figure 4A). Treatment of macrophages with simvastatin significantly decreased filipin intensity. Supplementation of mevalonate restored cholesterol content. Similar results were observed with uninfected macrophages. Interestingly, LM infection of macrophages resulted in significantly increased cholesterol levels when compared to uninfected cells (Figure 4B). Lipid extraction was further used to quantify the total cholesterol content in macrophage lysates. As expected from the filipin-staining assay, simvastatin or methyl- $\beta$ -cyclodextrin (M $\beta$ CD)-treated macrophages reduced the cholesterol content. The addition of mevalonate or exogenous cholesterol, respectively restored the cholesterol content (Figure 4C), demonstrating that simvastatin indeed decreases cellular cholesterol in macrophages.



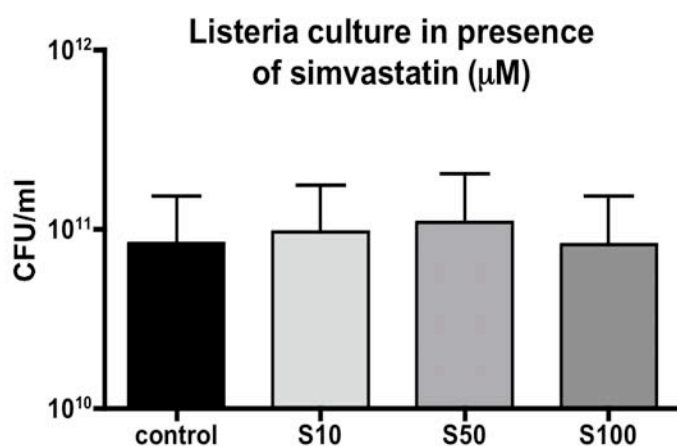
**Figure 4. Simvastatin decreased intracellular cholesterol levels.**

(A) Representative confocal images from simvastatin  $\pm$  mevalonate-treated macrophages were stained with filipin and the fluorescent intensity (arbitrary units) per cell was quantified by Laser Scanning Microscope (LSM) software. Data is shown as intensity from 25-45 cells/group. (B) Cholesterol levels were measured in naive and LM infected macrophages as indicated in (A). (C) Cholesterol content of macrophages was measured in cell lysates following lipid extraction by commercial kit. All data are shown as mean  $\pm$  SEM and are representative of two independent experiments, \*  $P < 0.05$ , \*\*  $P < 0.01$  compared to control.

## 2. The role of statins during *Listeria monocytogenes* infection

### 2.1 Simvastatin had no effect on extracellular growth of *L. monocytogenes* in culture broth

We next investigated whether simvastatin has a direct bactericidal effect on the growth of *L. monocytogenes* in culture medium. On the basis of the primary structure, sequence alignment and sensitivity to statins HMG-CoA reductase enzymes are classified into Class I and Class II (Bochar, Stauffacher et al. 1999). Eukaryotes utilizes class I HMG-CoA reductase enzymes which can be inhibited by statins at lower concentrations. In contrast, class II reductase enzymes are utilized by prokaryotes and its inhibition requires 1000-fold higher concentrations of statins than compared to class I enzymes (Hedl and Rodwell 2004). To test this hypothesis, we measured bacterial growth in culture medium containing different concentrations of simvastatin. No differences in bacterial growth were observed between control and simvastatin supplemented in the culture broth, indicating that simvastatin at the concentrations used for this study, has no direct effect on HMG-CoA reductase of LM. This result suggests that the observed reduced growth of LM in macrophages was not due to the direct effect of simvastatin on LM (Figure 5).

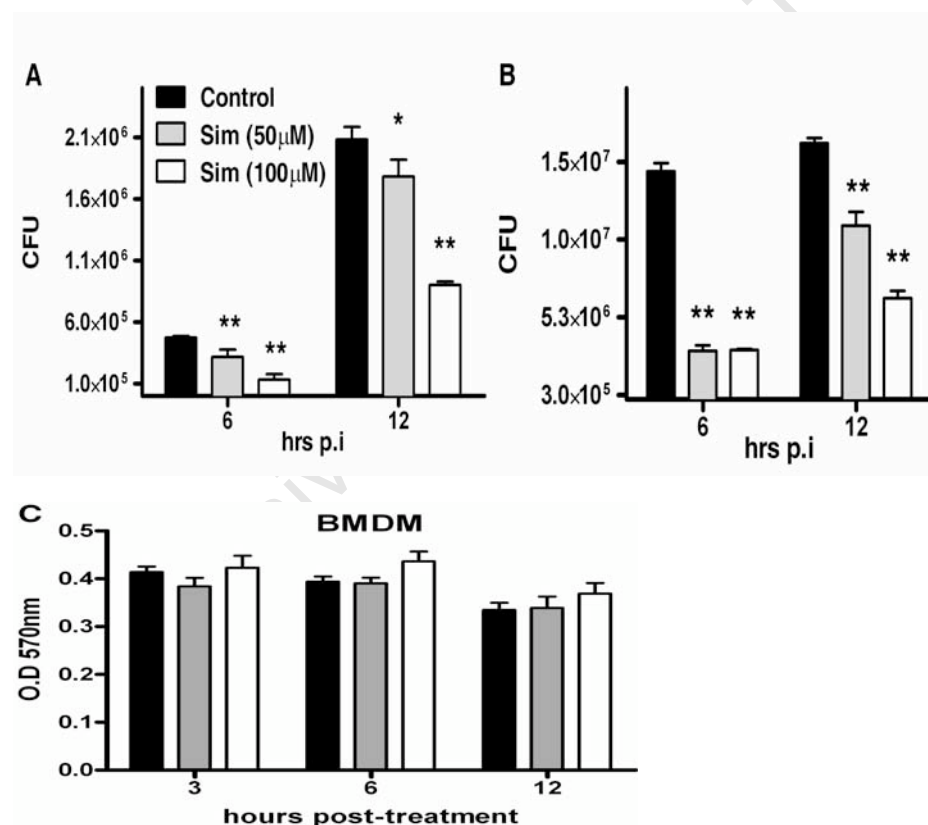


**Figure 5. Effect of simvastatin on growth of extracellular *Listeria monocytogenes*.**

*Listeria monocytogenes* were inoculated in tryptic soy broth medium containing indicated concentrations of simvastatin. Overnight cultures were then incubated on tryptic soy agar plates to determine viable colony counts. Data pooled from two independent experiments. All error bars indicate mean  $\pm$  SEM.

## 2.2 Simvastatin reduced intracellular growth of *L. monocytogenes* in macrophages

To investigate whether simvastatin could limit bacterial growth *in vitro*, murine primary macrophages (Figure 6A) and the macrophage cell line RAW264.7 (Figure 6B) were infected with LM and viable bacteria were determined at different time points post-infection. Growth of LM was significantly reduced in macrophages treated with simvastatin at 50 and 100  $\mu\text{M}$ . To rule out the possibility of simvastatin-induced cytotoxicity, cell viability was measured by a colorimetric enzymatic MTT assay. No major differences in cell viability were observed (Figure 6C). These results indicate that simvastatin decreases growth of LM in a dose-dependent manner and independent of cell type.



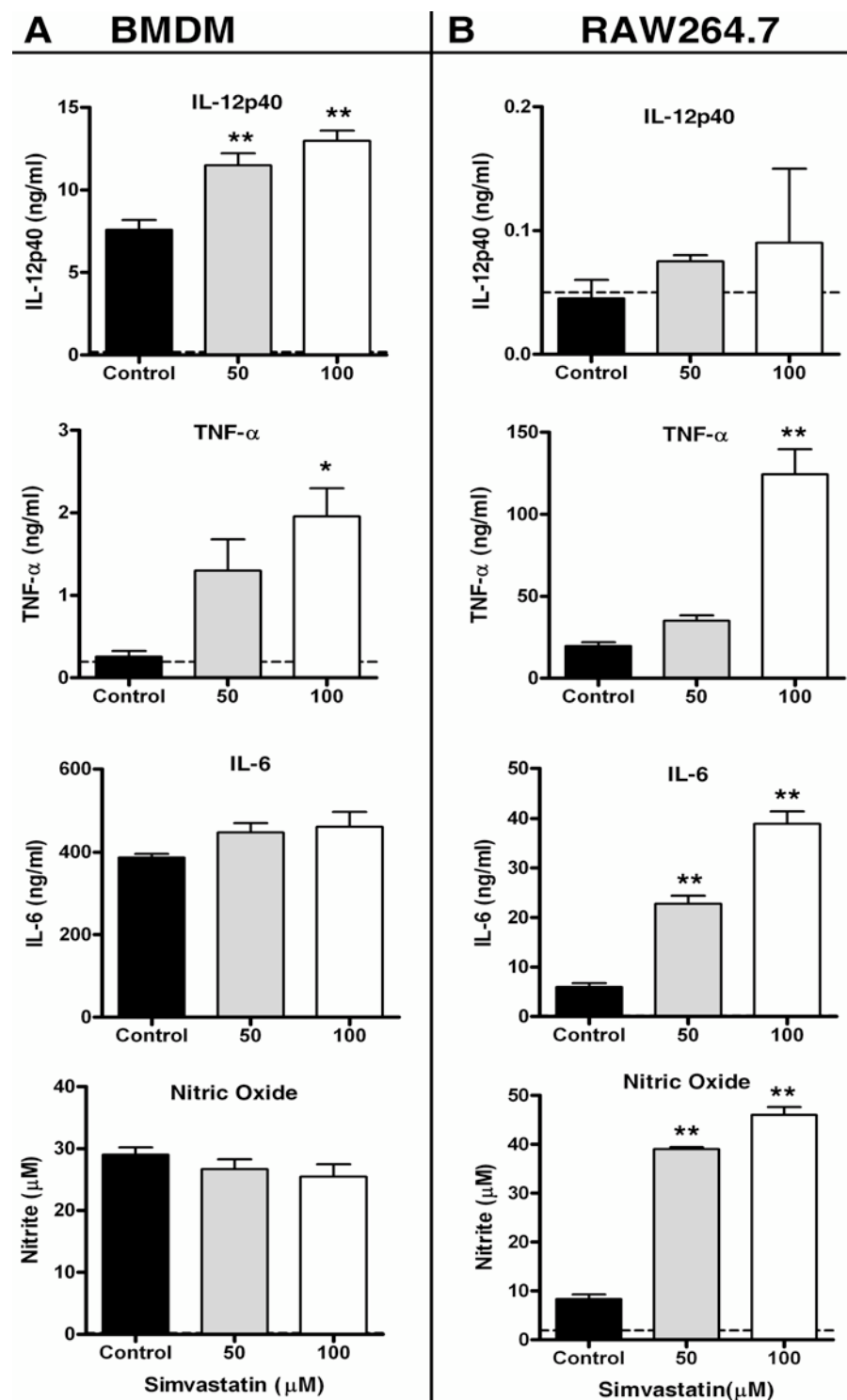
**Figure 6. Simvastatin decreased growth of *Listeria monocytogenes* in macrophages.**

(A) Murine bone marrow-derived macrophages and (B) RAW264.7 macrophage cell line were treated overnight with different concentrations of simvastatin and then infected with *L. monocytogenes* for 1 hour. After 1 hour of infection cells were washed and extracellular bacteria were removed by treating cells with gentamicin. Bacterial growth was measured at 6 and 12 hours post infection. (C) In parallel, BMDM were treated as mentioned above to measure cell viability by enzymatic MTT assay. Results are shown as mean  $\pm$  SEM of triplicate cultures, one-way ANOVA followed by Dunnett's post-test and are representative of two independent experiments, \*  $P < 0.05$ , \*\*  $P < 0.01$  compared to control.

### **2.3 Increased IL-12p40, TNF- $\alpha$ , IL-6 and nitric oxide production by macrophages following simvastatin treatment**

The effect of simvastatin on cytokine production was studied in murine bone marrow-derived macrophages (BMDM) and the RAW264.7 macrophage cell line. We pre-treated BMDM and RAW264.7 cells with simvastatin and IFN- $\gamma$  for 18 hours followed by infection with LM. At 12 hours post-infection, simvastatin enhanced the production of IL-12p40 in a dose-dependent manner which was accompanied by a modest increase in TNF- $\alpha$  and IL-6 levels, whereas nitric oxide production was slightly reduced in BMDM (Figure 7A), which is essential for Th1 response against bacterial pathogens. This finding is in line with a previous study in which simvastatin increased LPS-induced IL-12p40 and TNF- $\alpha$  production in peritoneal macrophages by mechanisms, involving AP-1 and C/EBP transcription factors (Matsumoto, Einhaus et al. 2004). In addition, there was reduced production of IL-12p70 at an early time point and although it was detected at a later time point no major difference was observed with simvastatin treated macrophages (data not shown). RAW264.7 macrophages exhibited a slightly altered cytokine profile when compared to BMDM, with significantly increased TNF, IL-6 and NO levels following simvastatin treatment. Simvastatin treatment had no significant effect on the levels of IL-12p40 (Figure 7B). The magnitude of the cytokine response is slightly altered in terms of the magnitude dependent upon the type of cell used. Altogether, these results suggest that the host protective effect of simvastatin was partially dependent on cytokines and nitric oxide mediated microbicidal activity in macrophages.



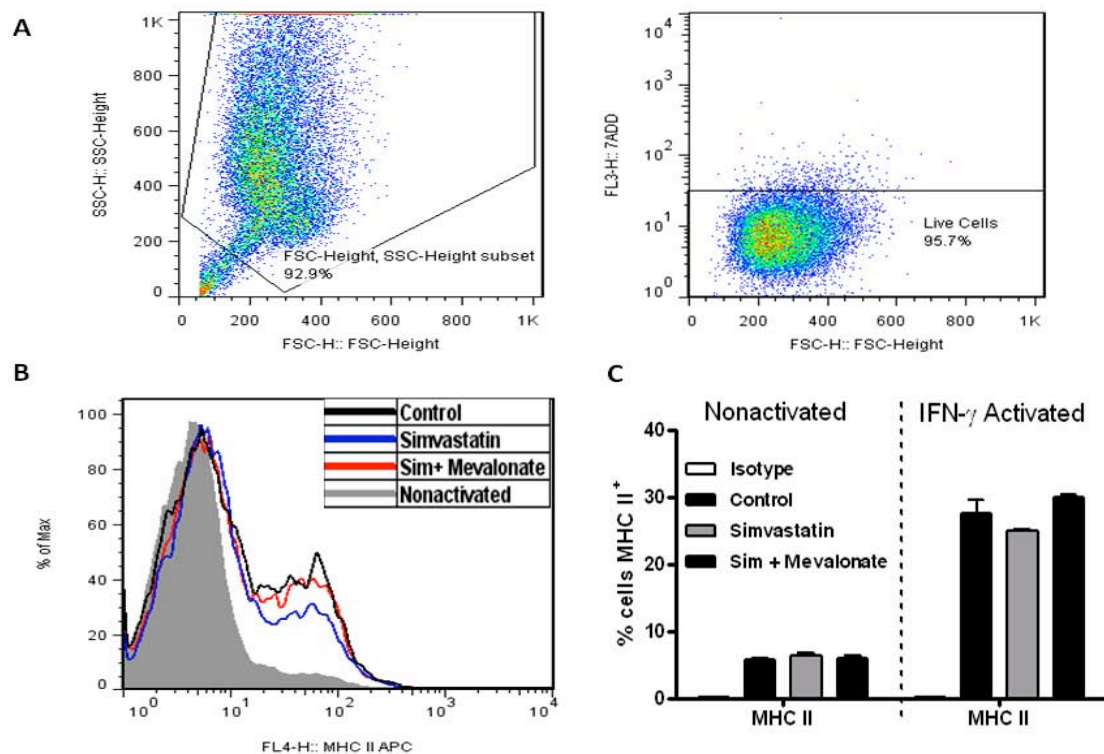


**Figure 7. Simvastatin mediated cytokine response and release of nitric oxide in primary macrophages and RAW264.7 cells following *Listeria monocytogenes* infection.**

(A) Murine bone marrow-derived macrophages and (B) RAW264.7 cells, a murine macrophage cell line, were treated overnight with the indicated concentrations of simvastatin and then infected with *L. monocytogenes*. After 12 hours, culture supernatants were harvested to measure cytokine release using ELISA. Results are shown as mean  $\pm$  SEM of triplicate cultures, dotted line reflects limit of detection. Data are analyzed by one-way ANOVA followed by Dunnett's post-test and are representative of two independent experiments, \*  $P < 0.05$ , \*\*  $P < 0.01$  compared to control.

#### **2.4 Simvastatin reduced IFN- $\gamma$ -driven MHC-II expression in macrophages**

Evidence indicates that statins decrease expression of MHC-II by IFN- $\gamma$  in several cell types, including human monocyte-derived macrophages. Mechanistically, statins down regulate the expression of inducible class II transactivator promoter IV (CIITA), causing reduction in MHC-II expression and thus decreasing T cell activation (Kwak, Mulhaupt et al. 2000). To investigate whether simvastatin regulates expression of MHC-II, macrophages were stimulated by IFN- $\gamma$  and infected with LM. The expression of MHC-II was decreased following treatment with simvastatin (Figure 8B). This could be reversed by adding exogenous mevalonate. These results are consistent with previous report, showing the abrogated affect of statins following mevalonate supplementation (Vaughan, Gotto et al. 2000). Furthermore, simvastatin had no effect on constitutive expression of MHC-II on macrophages (Figure 8C). This finding is in agreement with an earlier report, which showed that statins specifically target IFN- $\gamma$  induced expression of MHC-II without an effect on constitutive expression of MHC-II on human dendritic cells (DC) or B lymphocytes (Pedersen 1999). Together, these results suggest that simvastatin can modulate immune response by decreasing MHC-II expression induced by IFN- $\gamma$  in macrophages.



**Figure 8. Simvastatin decreased IFN- $\gamma$ -driven MHC-II expression by macrophages following *L. monocytogenes* infection.**

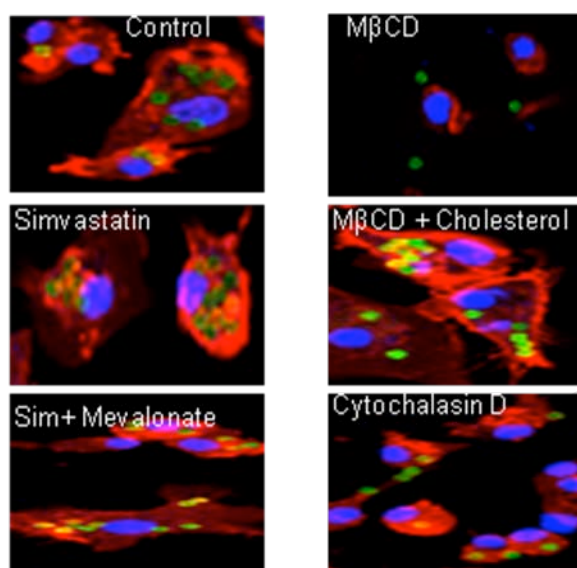
IFN- $\gamma$  activated or non-activated macrophages were treated overnight with 100 $\mu$ M simvastatin (red line) or simvastatin in combination with 100  $\mu$ M mevalonate (blue line). Macrophages were then infected with LM for 1 hour. Cells were washed and collected for flow-cytometric analysis of MHC-II protein expression. (A) Gating strategy and selection of live macrophages (B) MHC-II expression in non-activated (gray shade) and IFN- $\gamma$  activated macrophage in presence of simvastatin or simvastatin with mevalonate. (C) Quantification of MHC-II positive macrophage. Results are shown as mean  $\pm$  SEM of triplicate cultures and are representative of three independent experiments.

## 2.5 Simvastatin had no major effect on phagocytic ability of macrophages

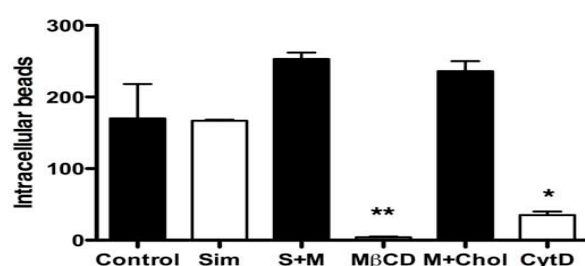
Membrane cholesterol plays a key role during infection by intracellular pathogens, such as viruses and protozoa (Simons and Ehehalt 2002; Manes, del Real et al. 2003). Methyl- $\beta$ -cyclodextrin (M $\beta$ CD), a cyclic oligosaccharide, selectively removes membrane cholesterol leading to conformational changes in membrane and receptors, thereby inhibiting downstream signal transduction (Kabouridis, Janzen et al. 2000). Thus, treating cultured cells with M $\beta$ CD has a severe effect on the membrane morphology by sudden sequestration of cholesterol and subsequent distortion of rafts. The severity of M $\beta$ CD treatment on the membrane was tested by macrophage internalization studies with green fluorescent labelled beads by confocal microscopy. Within 1 hour of incubation, internalization of beads was similar in control and simvastatin-treated

macrophages (Figure 9). In contrast, cell surface cholesterol depletion by M $\beta$ CD resulted in almost complete inhibition of phagocytosis, which was rescued by addition of external cholesterol. Cytochalasin D, a potent inhibitor of phagocytosis, served as a positive control to block internalization of beads. Taken together, these results suggest that M $\beta$ CD can severely impair non-specific phagocytosis, whereas simvastatin has no major effect on internalization of beads.

**A**



**B**

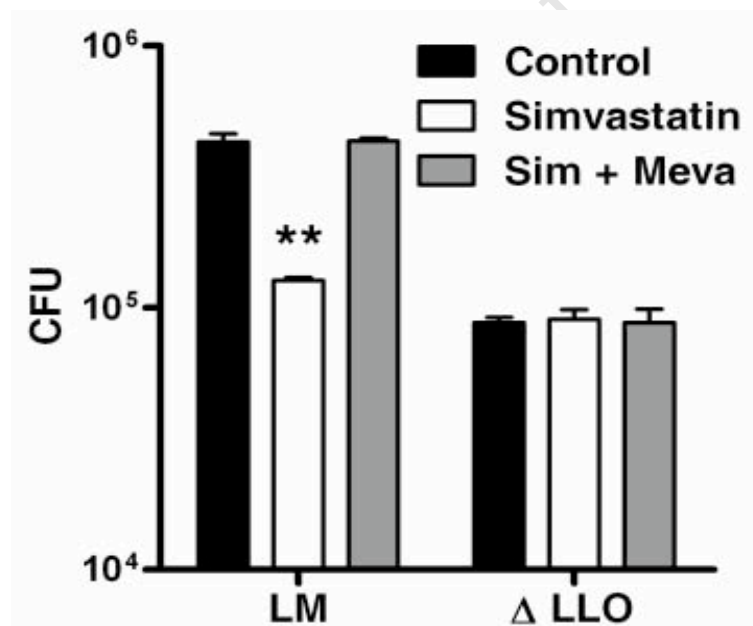


**Figure 9. Simvastatin had no effect on phagocytosis in macrophages**

(A) Macrophages were treated with either simvastatin  $\pm$  mevalonate or M $\beta$ CD  $\pm$  cholesterol for 24 hours, and then incubated with FITC (green) beads at MOI=10 to measure phagocytosis. Cells were stained with phalloidin rhodamine for actin (red) and nuclei were stained with DAPI (blue). (B) Quantification of the number of beads internalized from 50-100 macrophages/sample. All data are shown as mean  $\pm$  SEM and are representative of two independent experiments, \*  $P < 0.05$ , \*\*  $P < 0.01$  compared to control.

## 2.6 Statins decrease listerial growth in macrophages by interfering in LLO-dependent escape

Mevalonate is a precursor in the biosynthetic pathway of cholesterol downstream of HMG-CoA reductase. To determine if the effect on bacterial growth was a direct consequence of the cholesterol biosynthetic pathway inhibition, we added mevalonate to simvastatin-treated cells and restored cholesterol biosynthesis. Supplementation of mevalonate completely abrogated simvastatin-mediated decrease in bacterial growth (Figure 10). *Listeria* secretes a cholesterol-dependent listeriolysin (LLO) (Portnoy, Jacks et al. 1988; Tweten, Parker et al. 2001), crucial for bacterial escape into the cytoplasm. Interestingly, bacterial growth of a *L. monocytogenes* mutant strain lacking listeriolysin O (LLO) was neither affected by either treatment with simvastatin or mevalonate. Together, these results suggest that simvastatin provides protection against listeriolysin-mediated cytolysis. Hence, mevalonate-mediated cholesterol biosynthesis seems to play an important role for listerial growth within macrophages.

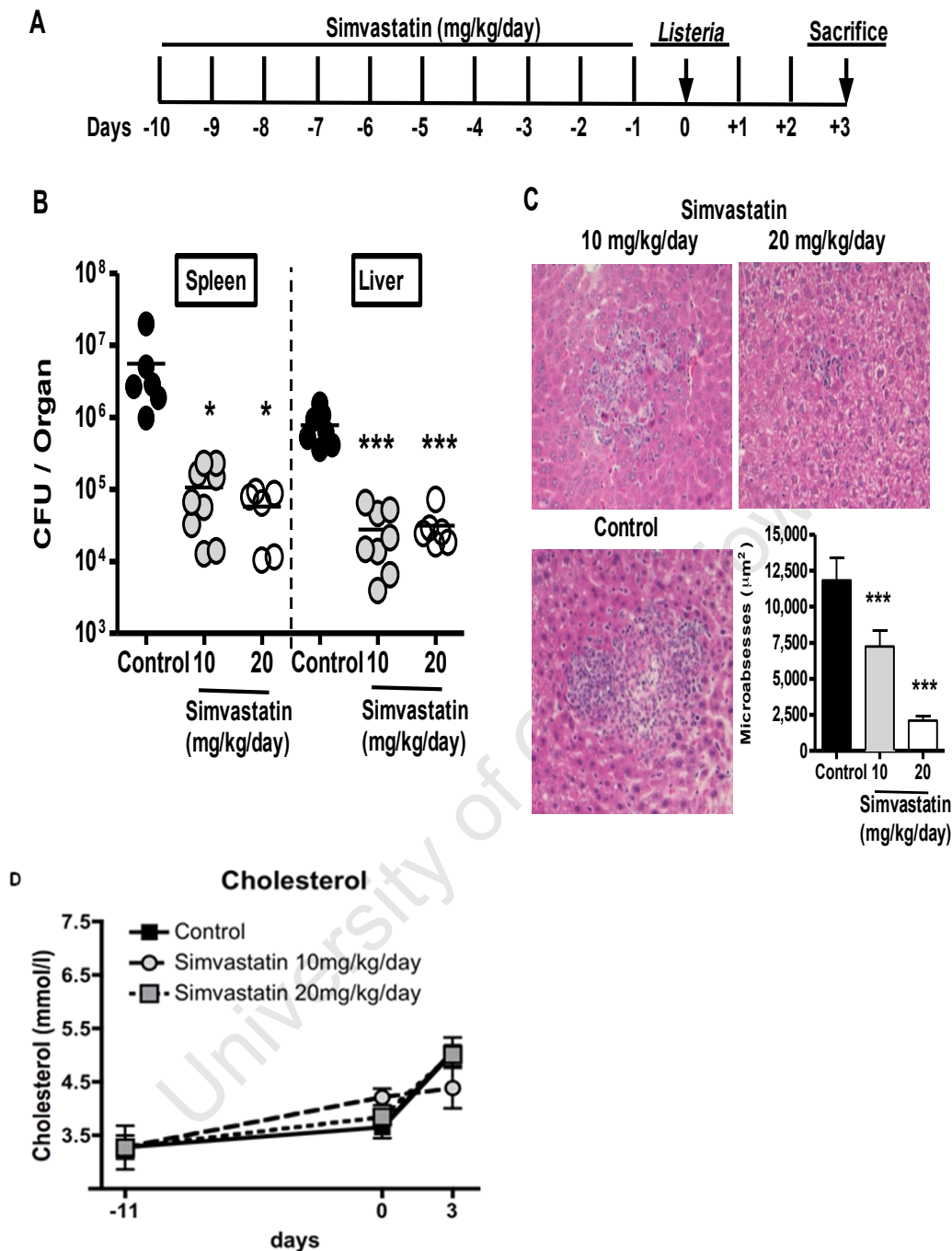


**Figure 10.** Simvastatin had no effect on growth of LLO-deficient *Listeria monocytogenes* in macrophages.

Macrophages were treated overnight with simvastatin ± mevalonate (100 μM each) and infected with either *L. monocytogenes* or LLO mutant LM. At 12 hours post infection, viable bacilli were determined. Results are shown as mean ± SEM of triplicate cultures, one-way ANOVA followed by Dunnett's post-test and are representative of three independent experiments, \*\*  $P < 0.01$  compared to control.

### 2.7 Simvastatin decreased *Listeria monocytogenes* burden in mice

To correlate the *in vitro* findings with *in vivo* effects in mice, we investigated the effect of simvastatin treatment on bacterial titers during the acute phase of LM infection. We treated adult mice with 10 and 20 mg/kg of simvastatin daily for 12 days by intraperitoneal injection as outlined in the layout (Figure 11A). Simvastatin treatment led to almost 2-log significant reduction of bacterial burden in both the liver and spleen (Figure 11B). This reduction in bacterial growth is accompanied by well-defined and small hepatic microabscesses in simvastatin-treated mice as compared to large microabscesses in control mice, revealed by liver histopathology and quantification of lesion sizes (Figure 11C). Serum cholesterol levels were not altered by the administration of statins. Interestingly, serum cholesterol levels were increased rapidly following LM infection (Figure 11D). Taken together, these results suggest that simvastatin is able to induce host protection against *Listeria monocytogenes* infection.



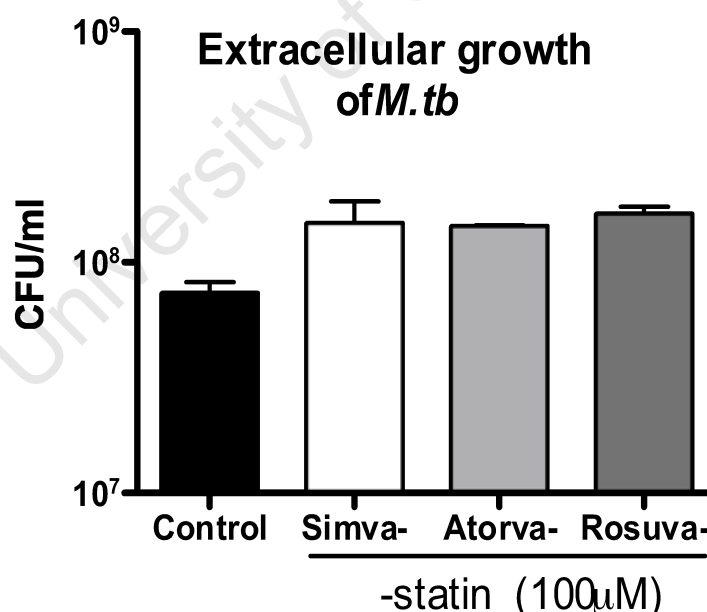
**Figure 11. *L. monocytogenes* burden is decreased in simvastatin-treated mice.**

C57BL/6 mice were treated with simvastatin at 10 or 20 mg/kg/day or with vehicle control by intraperitoneal injection for 12 days as indicated in layout (A). Mice were infected intraperitoneally with  $2 \times 10^5$  *L. monocytogenes* on day 11 and then sacrificed on day 14. (B) Bacterial burden were determined in spleen and liver at 3 days post infection. Data pooled from two independent experiments. (C) Representative image of liver histopathology from each group and lesion size was measured in 50-150 microabscesses/group from one experiment. (D) Serum cholesterol levels during the course of the infection and representative of two independent experiments. All error bars indicate mean  $\pm$  SEM, \*  $P < 0.05$ , \*\*\*  $P < 0.001$  compared to control, (n = 6-12 mice/group).

### 3. Role of statins during *Mycobacterium tuberculosis* infection

#### 3.1 The extracellular growth of *M. tuberculosis* is independent of statins in culture broth

Some evidence suggests that the *Mtb* genome contains an HMG-CoA reductase homolog (Lamb, Kelly et al. 1998; Bellamine, Mangla et al. 2001). However, other research groups were unable to identify this gene in *Mtb* (Cole, Brosch et al. 1998; Boucher and Doolittle 2000; Bailey, Mahapatra et al. 2002). It is noteworthy to mention that *Mtb* can utilize cholesterol and fatty acids as alternative carbon source to survive and persist in host (Van der Geize, Yam et al. 2007). Recently, it has been found that mammalian cell entry 4 (*mce4*) operon of *Mtb* is responsible for the uptake and metabolism of host lipids (Joshi, Pandey et al. 2006; Casali and Riley 2007). We addressed whether, simvastatin-mediated reduction in intracellular growth of *Mtb* was due to the direct influence of statins on the bacterial growth or host mediated response. Here, we show that the extracellular replication of *M. tuberculosis* in culture broth medium was not affected by supplementation of statins (Figure 12).



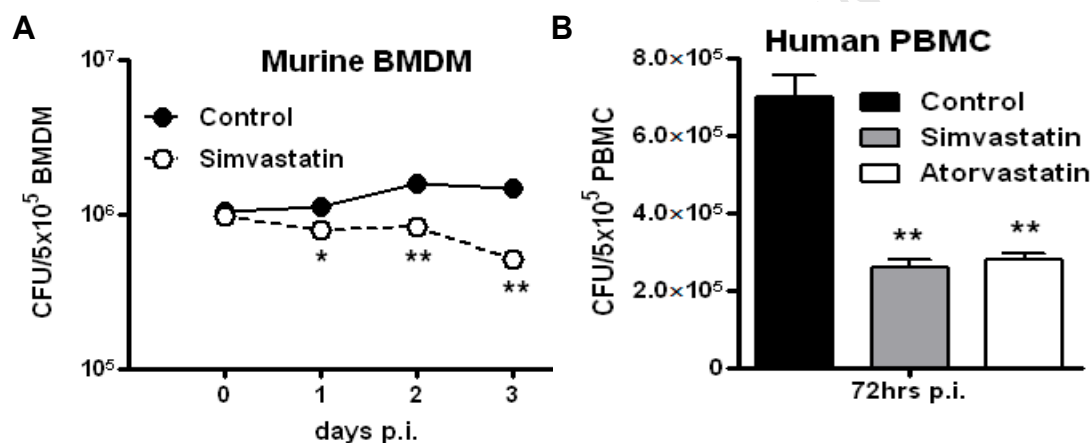
**Figure 12. Statin independent growth of *Mycobacterium tuberculosis* in culture broth.**

*M. tuberculosis* (H<sub>37</sub>Rv) was inoculated in 7H9 culture broth supplemented with different statins at 100µM. Cultures were then incubated for 12 days and plated on 7H10 agar plates to determine viable bacilli numbers. Results are expressed as CFU/ml and are representative of two independent experiments. All error bars indicate mean ± SEM.



### 3.2 Simvastatin reduce intracellular growth of *M. tuberculosis* in murine macrophages and human peripheral blood mononuclear cells (PBMC)

Next, we investigated whether treatment of cells with statins could potentially reduce the bacterial growth of *M. tuberculosis* (*Mtb*) in murine BMDM (Figure 13A) and human PBMC (Figure 13B). Growth of *Mtb* was clearly impaired from day 1 to day 3 post-infection over the course of infection in murine macrophages. Human PBMC were treated with two lipophilic statins that passively diffused across cellular membranes. We demonstrated that simvastatin and atorvastatin reduced the growth of *Mtb* in human PBMC. Altogether, we conclude that statin treatment induces protection against *M. tuberculosis* in macrophages.

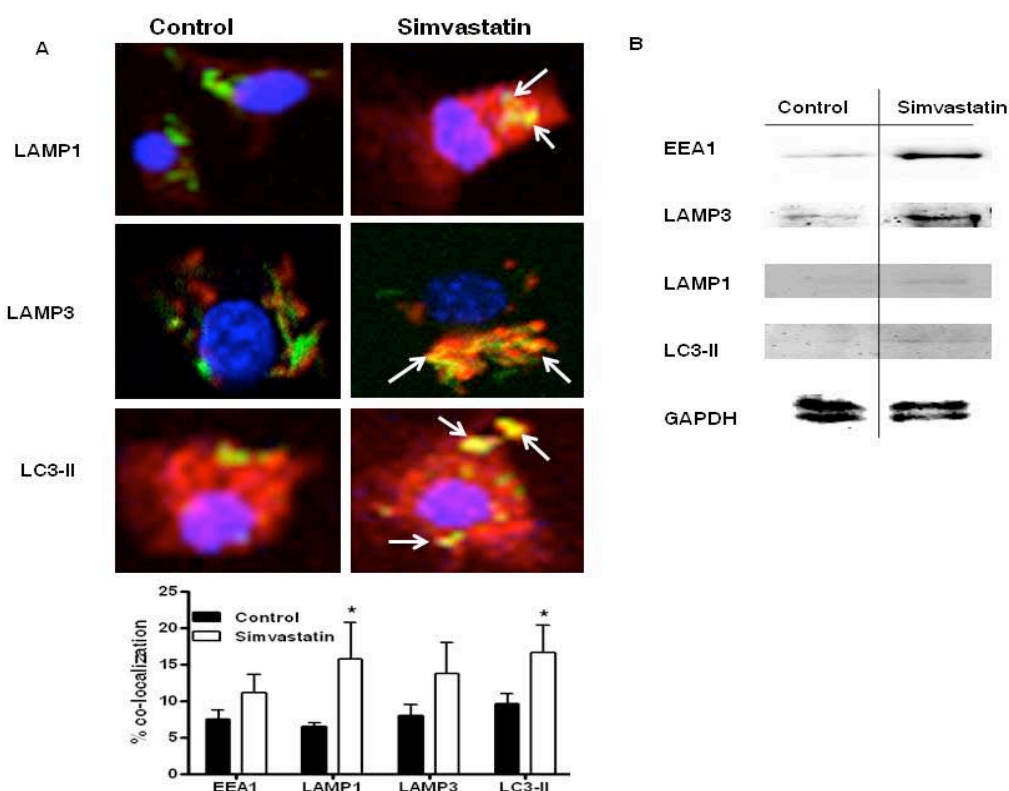


**Figure 13. Simvastatin decreased growth of *Mtb* in murine macrophages and human PBMC** (A) BMDM and (B) human PBMC were incubated overnight in the presence of statin or medium alone. Cells were then infected with *Mtb* at MOI=5 for 4 hours. After 4 hours of infection, cells were washed to remove extracellular bacteria and cultured with medium with 20ug/ml of gentamicin. At 72 hours post-infection, cells were lysed to determine viable bacterial counts. Results are shown as the mean  $\pm$  SEM of cultures in triplicates and representative of two independent experiments, \*  $P < 0.05$ , \*\*  $P < 0.01$  compared to control.

### 3.3 Statins promote maturation of phagosomes and induce autophagy

During the *Mtb* internalization process, bacteria associate with cholesterol rich membrane domains (Russell, Cardona et al. 2009). Cholesterol is reported to accumulate on endosomes in order to delay and inhibit phagolysosomal maturation (Manes, del Real et al. 2003). We tested whether the decrease in intracellular cholesterol levels due to statin treatment is able to reverse this process. In order to investigate this, simvastatin treated or untreated BMDM's were infected with GFP-*Mtb*. Two hours later, phagosomal maturation markers were labeled with fluorescent antibodies and co-localization studies were

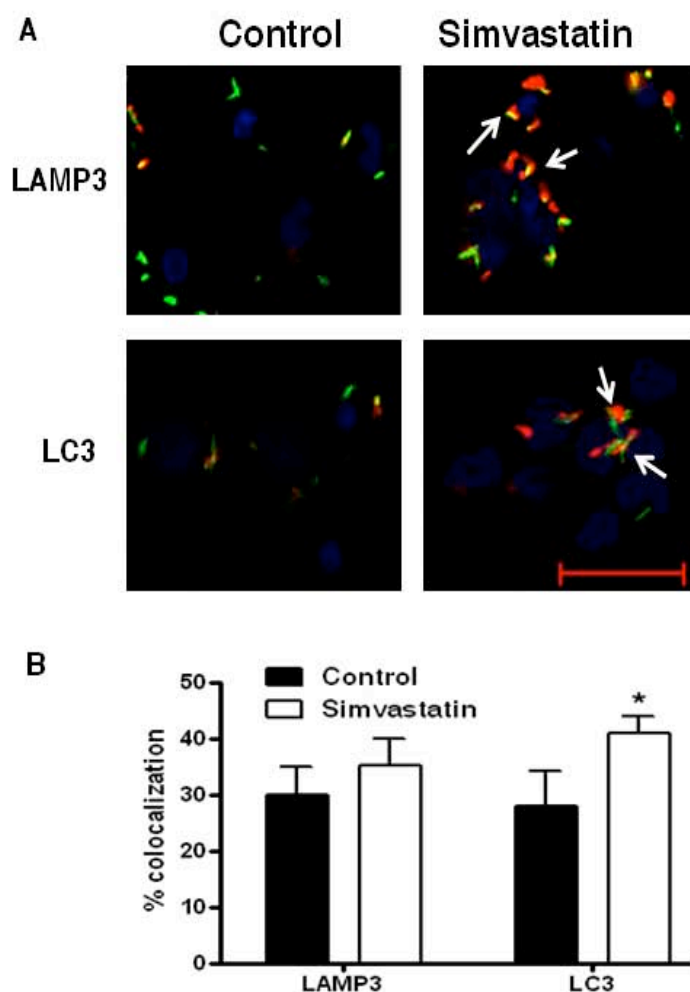
performed (Figure 14A). The early marker, early endosomal antigen 1 (EEA1) is recruited to phagosomes as soon as a pathogen is internalized. This is followed by sequential recruitment of late markers such as lysosomal-associated membrane protein 1 (LAMP1) and lysosomal-associated membrane protein 3 (LAMP3) to fuse with lysosomes. Simvastatin-treated macrophages showed increased co-localization with EEA1, LAMP1 and LAMP3. Only expression of LAMP1 reached statistical significance when compared to control macrophages. The increase of the markers EEA1, LAMP1 and LAMP3 was confirmed by Western blot (Figure 14B). Together, these results suggest that the influence of statins on cholesterol may counteract *Mtb*-induced inhibition of phagosomal maturation.



**Figure 14. Enhanced phagosomal maturation and autophagy following simvastatin treatment in murine macrophages.**

Macrophages were treated with simvastatin (100  $\mu$ M) overnight, followed by infection with GFP-*Mtb* at MOI of 10. After 2 hours of infection, cells were washed and stained with antibodies against phagosomal (LAMP1/LAMP3) or autophagy (LC3-II) markers (red) and nuclei were stained with DAPI (blue). (A) Representative confocal images and quantification of co-localization. Quantitative analysis of co-localization of GFP-*Mtb* with phagosomal or autophagy markers were generated from 4 fields/replicate consisting of 600 cells/sample in BMDM. (B) Detection of indicated markers by Western blot analysis in *Mtb* infected whole cell lysates. Results are shown as mean  $\pm$  SEM from duplicate samples and representative of three independent experiments, \*  $P < 0.05$  compared to control.

As a survival mechanism, infected macrophages are able to induce autophagy to bypass *Mtb*-mediated inhibition of phagolysosomal maturation and subsequent persistence (Gutierrez, Master et al. 2004; Singh, Davis et al. 2006; Jagannath, Lindsey et al. 2009). Induction of autophagy leads to the conjugation of soluble light chain 3-I (LC3-I) with phosphatidylethanolamine, which then incorporate into the autophagic membrane as light chain 3-II (LC3-II). This facilitates fusion of the autophagosome with the lysosomes (Rabinowitz and White 2010). Statin treatment before *Mtb* infection resulted in significantly increased co-localization of the bacteria with LC3-II in BMDM (Figure 14A), confirmed by Western blot analysis (Figure 14B) and also observed in co localization studies performed with human peripheral blood mononuclear cells (PBMC) (Figure 15). These results suggest that the influence of statins on cholesterol may induce host-mediated autophagy during *Mtb* infection.

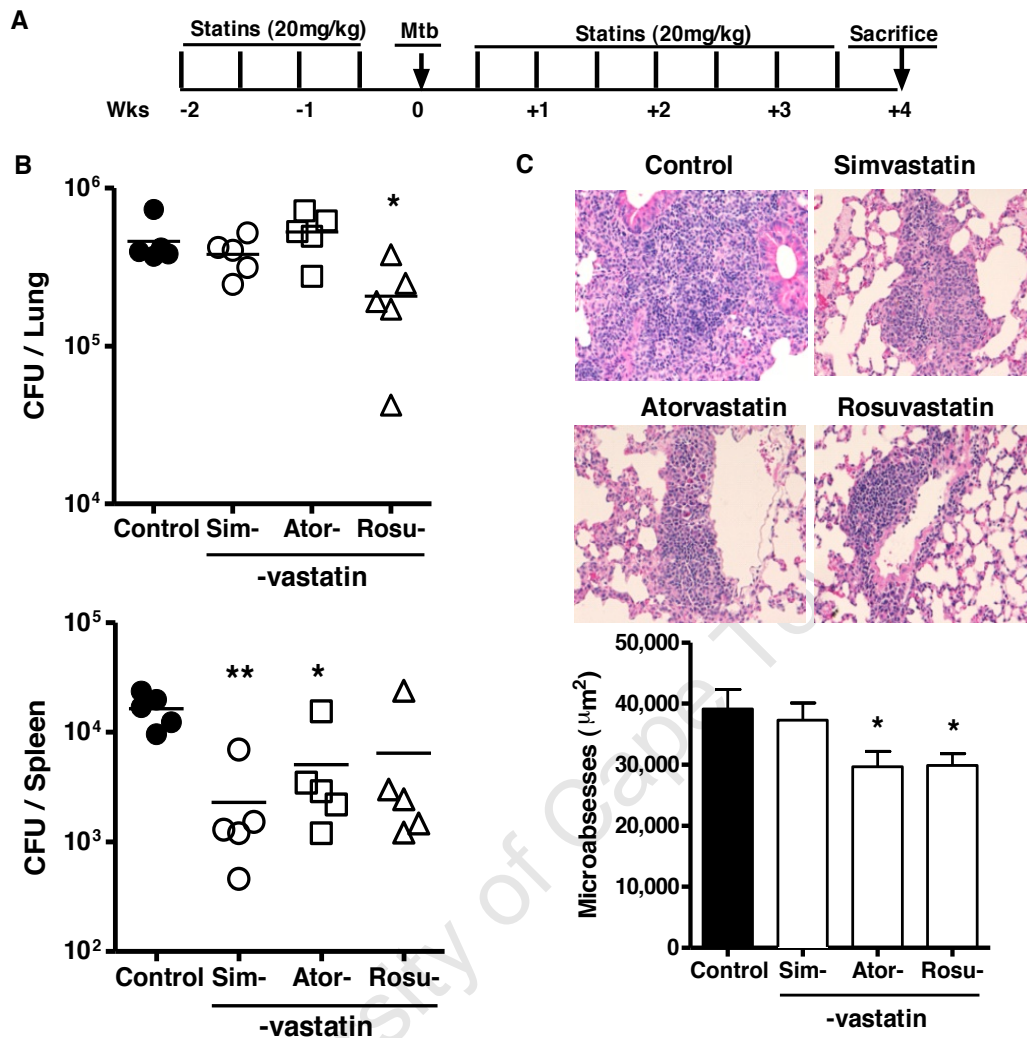


**Figure 15. Phagosomal maturation and enhanced autophagy following simvastatin treatment in human PBMC.**

Human PBMC were treated with simvastatin (100  $\mu$ M) overnight, followed by infection with GFP-*Mtb* at MOI of 10. After 2 hours of infection, cells were washed and stained for antibodies against either LAMP3 or LC3-II (red) and nuclei were stained with DAPI (blue). (A) Representative confocal images and (B) Quantitative analysis of co-localization of GFP-*Mtb* with phagosomal or autophagy markers were generated from 2 fields/replicate consisting of 200 cells/sample in PBMC, scale bar= 20  $\mu$ m. Results are shown as mean  $\pm$  SEM from duplicate samples. Data are representative of two independent experiments, \*  $P < 0.05$  compared to control.

### **3.4 Statins mediated protection against *M. tuberculosis* infection in mice**

To determine whether statin treatment could provide protection against *M. tuberculosis* infection, mice were treated with different statins (20 mg/kg) every second day for 6 weeks as shown in the layout (Figure 16A). Intraperitoneal injection of simvastatin, atorvastatin and rosuvastatin resulted in a significant 1-log reduction in bacilli burden in the spleen but not in the lungs, where only rosuvastatin achieved a significant reduction (Figure 16B). The intraperitoneal route of statin injection might more effectively reach the spleen than the lungs, explaining the discrepancies in burden observed between those two organs. Furthermore, reduced bacterial burden in lungs was supported by smaller microabscesses in statin-treated mice compared to control, revealed by lung histopathology and quantification of lesion size (Figure 16C). These results suggest that treatment of mice with statins results in reduced dissemination of mycobacteria into the spleen following aerosol infection with *Mtb*.

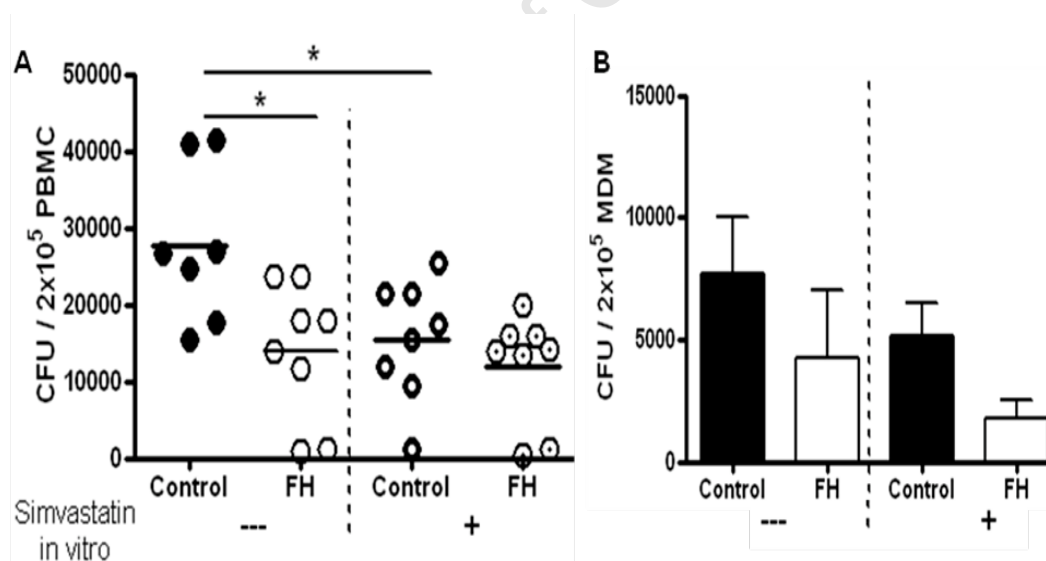


**Figure 16. Decreased *Mtb* dissemination and reduced lung inflammation in statin-treated mice.**

(A) C57BL/6 mice were treated for 6 weeks with the indicated statins (20mg/kg q.o.d) or control by intraperitoneal injection. Mice were infected via aerosol inhalation with *M. tuberculosis*, H37Rv (400 CFU/mouse) as indicated in the layout. (B) Bacterial titers were determined in the spleen and lungs at 4 weeks post-infection. (C) Representative images of lung pathology stained for haematoxylin/eosin. Original magnification, X200. Lung lesion size was quantified in 45-60 microabscesses/group. Data are represented as mean  $\pm$  SEM of one experiment (n = 5 mice/group), \*  $P < 0.05$ , \*\*  $P < 0.01$  compared to control.

### 3.5 Mononuclear cells and macrophages derived from patients on statin therapy are less susceptible to *Mycobacterium tuberculosis* infection

Familial hypercholesterolemia (FH) patients with high serum LDL-cholesterol levels are daily treated with statins. To assess the impact of statin therapy on inducing host protective immunomodulatory functions against tuberculosis, we evaluated the bacterial growth of *Mtb* in human peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM) from patients with FH and healthy non-statin users. Growth of *Mtb* in PBMC from statin-treated patients was significantly reduced by almost 3-fold when compared to PBMC from healthy controls (Figure 17A). In addition, mycobacterial growth was also reduced to the same magnitude in mononuclear cells of healthy donors following pre-treatment with statin *in vitro*. Similarly, results were observed in MDM (Figure 17B). These results show that statin-exposed PBMC have acquired protective immune responses that can lead to reduced bacterial growth following subsequent infection with *Mtb* *in vitro*.



**Figure 17 Reduced growth of *Mtb* in human mononuclear cells and macrophages derived from statin-treated patients with familial hypercholesterolemia (FH).**

(A) PBMC and (B) MDM from healthy donors and FH patients were isolated using citrate cell preparation tubes (CPT). Mononuclear cells and macrophages were incubated overnight in presence of either simvastatin or medium alone. Cells were then infected with *Mtb* at MOI=5 (Mononuclear cells) and MOI=2 (MDM) for 4 hours. After 4 hours of infection, cells were washed to remove extracellular bacteria and cultured in medium with 25 µg/ml gentamicin. At 72 hours post-infection, cells were lysed to determine viable bacterial counts. Results are shown as the mean ± SEM of n=8 donors/group (PBMC) and n=2 donors/group (MDM), \*  $P < 0.05$  compared to control.

In summary, we investigated the role of statin treatment in experimental murine models for human listeriosis and tuberculosis. As mice showed increased resistance against both diseases, the underlying mechanism was investigated. Statins reduced listerial growth in macrophages by counteracting *Listeria*-induced increased cholesterol levels, interfering in listeriolysin-O-dependent phagosomal escape into the cytoplasm. Statin treatment also reduced bacterial growth in *Mycobacterium*-infected murine and human macrophages, as well as in peripheral blood mononuclear cells (PBMC) isolated from patients with familial hypercholesterolemia (FH) on statin therapy when compared to healthy untreated subjects. Decrease of intracellular cholesterol levels by statins counteracted *Mycobacterium*-induced inhibition of phagosomal maturation and increased host-protective autophagy.

#### 4. References

- Bailey, A. M., S. Mahapatra, et al. (2002). "Identification, cloning, purification, and enzymatic characterization of Mycobacterium tuberculosis 1-deoxy-D-xylulose 5-phosphate synthase." *Glycobiology* **12**(12): 813-820.
- Bellamine, A., A. T. Mangla, et al. (2001). "Structural requirements for substrate recognition of Mycobacterium tuberculosis 14  $\alpha$ -demethylase: implications for sterol biosynthesis." *J Lipid Res* **42**(1): 128-136.
- Bochar, D. A., C. V. Stauffacher, et al. (1999). "Sequence comparisons reveal two classes of 3-hydroxy-3-methylglutaryl coenzyme A reductase." *Mol Genet Metab* **66**(2): 122-127.
- Boucher, Y. and W. F. Doolittle (2000). "The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways." *Mol Microbiol* **37**(4): 703-716.
- Casali, N. and L. W. Riley (2007). "A phylogenomic analysis of the Actinomycetales mce operons." *BMC Genomics* **8**: 60.
- Cole, S. T., R. Brosch, et al. (1998). "Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence." *Nature* **393**(6685): 537-544.
- Denoyelle, C., P. Albanese, et al. (2003). "Molecular mechanism of the anti-cancer activity of cerivastatin, an inhibitor of HMG-CoA reductase, on aggressive human breast cancer cells." *Cell Signal* **15**(3): 327-338.
- Gaillard, J. L., P. Berche, et al. (1991). "Entry of L. monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci." *Cell* **65**(7): 1127-1141.
- Glynn, S. A., D. O'Sullivan, et al. (2008). "The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, simvastatin, lovastatin and mevastatin inhibit proliferation and invasion of melanoma cells." *BMC Cancer* **8**: 9.
- Gutierrez, M. G., S. S. Master, et al. (2004). "Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages." *Cell* **119**(6): 753-766.
- Hamelin, B. A. and J. Turgeon (1998). "Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors." *Trends Pharmacol Sci* **19**(1): 26-37.
- Hedl, M. and V. W. Rodwell (2004). "Inhibition of the class II HMG-CoA reductase of Pseudomonas mevalonii." *Protein Sci* **13**(6): 1693-1697.
- Jagannath, C., D. R. Lindsey, et al. (2009). "Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells." *Nat Med* **15**(3): 267-276.
- Joshi, S. M., A. K. Pandey, et al. (2006). "Characterization of mycobacterial virulence genes through genetic interaction mapping." *Proc Natl Acad Sci U S A* **103**(31): 11760-11765.
- Kabouridis, P. S., J. Janzen, et al. (2000). "Cholesterol depletion disrupts lipid rafts and modulates the activity of multiple signaling pathways in T lymphocytes." *Eur J Immunol* **30**(3): 954-963.
- Kita, T., M. S. Brown, et al. (1980). "Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in livers of mice treated with mevinolin, a competitive inhibitor of the reductase." *J Clin Invest* **66**(5): 1094-1100.



- Kwak, B., F. Mulhaupt, et al. (2000). "Statins as a newly recognized type of immunomodulator." *Nature medicine* **6**(12): 1399-1402.
- Lamb, D. C., D. E. Kelly, et al. (1998). "A sterol biosynthetic pathway in *Mycobacterium*." *FEBS Lett* **437**(1-2): 142-144.
- Manes, S., G. del Real, et al. (2003). "Pathogens: raft hijackers." *Nat Rev Immunol* **3**(7): 557-568.
- Matsumoto, M., D. Einhaus, et al. (2004). "Simvastatin augments lipopolysaccharide-induced proinflammatory responses in macrophages by differential regulation of the c-Fos and c-Jun transcription factors." *Journal of immunology* **172**(12): 7377-7384.
- Pedersen, T. R. (1999). "Statin trials and goals of cholesterol-lowering therapy after AMI." *Am Heart J* **138**(2 Pt 2): S177-182.
- Portnoy, D. A., P. S. Jacks, et al. (1988). "Role of hemolysin for the intracellular growth of *Listeria monocytogenes*." *The Journal of experimental medicine* **167**(4): 1459-1471.
- Rabinowitz, J. D. and E. White (2010). "Autophagy and metabolism." *Science* **330**(6009): 1344-1348.
- Russell, D. G., P. J. Cardona, et al. (2009). "Foamy macrophages and the progression of the human tuberculosis granuloma." *Nat Immunol* **10**(9): 943-948.
- Simons, K. and R. Eehalt (2002). "Cholesterol, lipid rafts, and disease." *J Clin Invest* **110**(5): 597-603.
- Singh, S. B., A. S. Davis, et al. (2006). "Human IRGM induces autophagy to eliminate intracellular mycobacteria." *Science* **313**(5792): 1438-1441.
- Tweten, R. K., M. W. Parker, et al. (2001). "The cholesterol-dependent cytolysins." *Current Topics in Microbiology and Immunology* **257**: 15-33.
- Van der Geize, R., K. Yam, et al. (2007). "A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into *Mycobacterium tuberculosis* survival in macrophages." *Proc Natl Acad Sci U S A* **104**(6): 1947-1952.
- Vaughan, C. J., A. M. Gotto, Jr., et al. (2000). "The evolving role of statins in the management of atherosclerosis." *J Am Coll Cardiol* **35**(1): 1-10.

# **Chapter 4**

## **Discussion**

University of Cape Town

## Discussion

Statins are a family of drugs widely used for the treatment of patients with hypercholesterolemia and cardiovascular diseases (Shepherd, Cobbe et al. 1995; Itakura, Kita et al. 2008). Observational studies among human statin users revealed intriguing outcomes on infectious diseases. For instance, US veterans on statin therapy were less susceptible to tuberculosis as compared to non-statin users. This study offers an advantage of being studied in real time based on actual physician and patient behavior that reflects natural settings rather than artificial settings of randomized clinical trials. (Cirillo 2008). Recently, a meta-analysis of sixteen cohorts on statin therapy revealed reduced mortality in patients to various bacterial infections (Tleyjeh, Kashour et al. 2009). The use of statins had a beneficial survival effect in patients suffering from bacteremia (Liappis, Kan et al. 2001; Thomsen, Hundborg et al. 2006). Furthermore, beneficial effect of statins were also reported on the outcome of parasitic (Coppens, Sinai et al. 2000; Parquet, Briolant et al. 2009), viral (Rothwell, Lebreton et al. 2009) and bacterial infections such as *Salmonella* (Catron, Lange et al. 2004) and *Streptococcus pneumonia* in mice (Rosch, Boyd et al. 2010).

The microbicidal potential of statins might depend on the solubility and permeability through cellular membrane, as lipophilic statins can more easily cross cell membranes, when compared to hydrophilic statins. We therefore used lipophilic simvastatin for both *in vivo* and *in vitro* experiments, which can penetrate cell membranes more effectively than its hydrophilic counterpart, pravastatin (Hamelin and Turgeon 1998). Previous published studies used the activated form of drugs, as opposed to the lactone form in the mouse model of inflammatory arthritis (Leung, Sattar et al. 2003). The rationale for statin use as lactone form in the present study was to better adhere to scenario that reflect current practice in human patients, as statins are administered in lactone form to humans, as it increases the selectivity of statins for their intended target organ the liver. This approach improves the efficacy of the drug as well as decreases systemic availability, which in turn avoids organ or tissue-specific toxicity, which might be attributed to potential side effects emerging in long-term therapy (Weitz-Schmidt, Welzenbach et al. 2001). Recently, both lovastatin

and simvastatin, when used in the lactone form have been shown to inhibit cholesterol biosynthesis in human monocyte-derived macrophages (Kempen, Vermeer et al. 1991). Moreover, mevalonate-mediated reversal of simvastatin-induced inhibition of cholesterol biosynthesis in our study reflects that the lactone form was indeed converted into the hydroxyl acid form by macrophages.

The first part of the study aims to establish the dose of statin and investigate possible drug-induced cytotoxicity on macrophages. The doses of statins were determined on the basis of previous studies in mice and rats used in the range of 10-100mg/kg/day (Mundy, Garrett et al. 1999; Ni, Egashira et al. 2001). We compared 2 to 20mg/kg/day dose range of statin in mouse model of LM infection. Statin treatment reduced bacterial burden in organs, when administered with 20mg/kg/day, a dose that reflects maximum inhibition of HMG-CoA reductase activity. At the doses tested, plasma cholesterol levels remained significantly unaltered *in vivo*, consistent with the previous finding that showed rodents express less LDL receptors than humans, resulting in decreased uptake of LDL cholesterol from the blood circulation (Krause and Princen 1998). A published study has shown that higher doses of statins are required in rodents as compared to humans because in rodents lower doses of statins highly up-regulate the activity of the target enzyme, HMGCo-A reductase (Kita, Brown et al. 1980).

In addition to statins, methyl- $\beta$ -cyclodextrin (M $\beta$ CD), another cholesterol depleting agent was tested. Treating cultured cells with M $\beta$ CD had severe effects on the membrane morphology by sudden sequestration of cholesterol, which would explain the negligible number of beads internalized in our study. The results presented here demonstrate that simvastatin was able to decrease cellular cholesterol in macrophages. Interestingly, this was as efficient as M $\beta$ CD treatment, furthermore addition of mevalonate or exogenous cholesterol was able to restore the cholesterol content of simvastatin- or M $\beta$ CD-treated macrophages. Hence, the profound effect of M $\beta$ CD posed a limitation on the dynamics of cellular cholesterol homeostasis, cell viability and proliferation. A previous study showed M $\beta$ CD mediated cholesterol depletion from the plasma membranes led to inhibition of clathrin-dependent endocytosis by 50%,

indicating that cholesterol is required for the formation of clathrin-coated vesicles and important for endocytosis (Rodal, Skretting et al. 1999). Reduced membrane cholesterol level may influence phagocytic uptake, which would explain the observed reduced bacterial burden in macrophages (Gatfield and Pieters 2000).

Accordingly, we next perform experiments to test the influence of statin on phagocytosis. In these experiments, we observed no effect on phagocytosis of beads and bacterial pathogens or major effect on cell viability in simvastatin-treated cells. Our results suggest that statin-mediated reduced bacterial growth was not due to impaired uptake or decreased cellular viability. Furthermore, in the present study we demonstrate that M $\beta$ CD result in inhibition of non-specific uptake of beads as opposed to statins. The effect of statins on phagocytosis however, remains elusive, as it has been reported to either decrease (Loike, Shabtai et al. 2004; Yilmaz, Reiss et al. 2006) or increase phagocytosis (Djaldetti, Salman et al. 2006; Salman, Bergman et al. 2008) in primary cells and in cell lines. In addition to phagocytosis, previous studies have shown that statins are able to inhibit cell proliferation in various cell types, including aggressive cancer cells (Denoyelle, Albanese et al. 2003), one possible explanation behind the significant drop in cell viability and proliferation reported in other studies could be due to lower efficacy of statins in cell lines (Rothwell, Lebreton et al. 2009). Mechanistically, inhibitory effect on cell proliferation was shown by statin-mediated blocking of G1/S transition phase of cell cycle via induction of cyclin-dependent-kinase (CDK) inhibitors p21 and p27 (Kikuchi, Nagata et al. 1997; Negre-Aminou, van Vliet et al. 1997; Ukomadu and Dutta 2003; Glynn, O'Sullivan et al. 2008).

Moreover, the route of LM infection in mice can alter the severity of infection. We performed survival study during LM infection, where infections were initiated by inoculating mice into the peritoneal cavity or orally. The peritoneal route of LM infection was preferred as opposed to the oral route, although the later is a natural route of LM infection in humans. This is due to the fact that, the oral route of infection in experimental models poses a limitation for the lack of reproducibility in achieving LD<sub>50</sub> and necessitates higher inoculum ( $10^7$ - $10^9$ ) to

establish infection in mice. This discrepancy on reproducibility of oral infection in mice is attributed to the lack of murine E-cadherins, which mediates adherence to host cells by interaction with listerial InlA, (Gaillard, Berche et al. 1991; Lecuit, Vandormael-Pournin et al. 2001), which facilitates uptake into non-phagocytic cells such as intestinal epithelial cells by altering local cytoskeletal rearrangement.

In the second part of the study, we investigated the role of the statins during LM infection *in vivo*, where simvastatin-treated mice showed nearly 2-log reduced bacterial titers of *L. monocytogenes* in spleen and liver. The decrease in bacterial growth was accompanied by smaller, well defined and fewer granulomatous lesions, revealed by liver histopathology. Here, we report for the first time that statins induce a host protective immunity against LM infection in mouse model of acute listeriosis that had not been previously published. This finding correlates with a previously published study showed that the subcutaneous treatment of atorvastatin resulted in 2 to 3-fold reduction in bacterial load of *Salmonella enterica*-infected mice (Catron, Lange et al. 2004). However, the increased protection in this study was likely contributed by the duration of the statin treatment, intraperitoneal route of drug administration and other bacterial disease model investigated. Subsequently, a decrease in LM growth was also confirmed in BMDM and the murine macrophage cell line RAW264.7 *in vitro*, indicating that growth inhibitory effect of statin was not dependent on the cell type. Importantly, the inhibitory effect of simvastatin on bacterial growth was reversed by the addition of mevalonate, the immediate downstream product synthesized by HMG-CoA.

To understand the mechanism by which statins inhibits LM growth in detail, we explored whether statins were able to protect macrophages from a cytolysin expressed by LM, listeriolysin O (LLO). This hypothesis was tested in macrophages infected with LM deficient for LLO ( $\Delta$ LLO). The growth of  $\Delta$ LLO was similar in control and simvastatin-treated macrophages, indicating that statins prevent LLO-mediated cytolysis when infected with wild type LM, and in the absence of LLO, this protective effect of statin was lost. These results suggest that simvastatin treatment in LM infected macrophages reduced intracellular cholesterol and therefore resulted in decreased bacterial growth in these cells,

which is contingent on cholesterol-dependent LLO. This suggests that statins counteract LLO-dependent escape into the cytoplasm. LLO, a major cholesterol-dependent cytolysin (CDC) expressed by LM required for escape from vacuole into the cytoplasm (Soltani, Hotze et al. 2007) to proliferate and disseminate into the neighbouring cells via actin comets or tails. This finding was consistent with a recent report showed that simvastatin treatment on endothelial cells protects from pneumolysin, a cytolysin secreted by *Streptococcus pneumoniae* (Rosch, Boyd et al. 2010).

The antimicrobial activity of statins could also be attributed to their immunomodulatory effects, as they have been reported to exhibit immunomodulatory functions (Kwak, Mulhaupt et al. 2000). Statins induced Th1 cytokines in human peripheral blood mononuclear cells (PBMC), where simvastatin and lovastatin induced the production of IL-1 $\beta$  in a dose-dependent manner. On the other hand, high concentrations of statins reduced the release of IL-1R $\alpha$  and IFN- $\gamma$ . In another study, statins showed inhibitory effects on the production of IL-2 in a dose-dependent manner (Montero, Hernandez et al. 2000). Statins were also able to polarize immune responses towards Th2 cytokines Graft-Versus-Host-Diseases (GVHD), where statin-mediated protection to GVHD was partially dependent on Stat6 phosphorylation and another recent study reported that mice treated with atorvastatin or fluvastatin increases IL-4 and IL-10 producing CD4<sup>+</sup> T-cells. In addition, serum IFN- $\gamma$  and TNF- $\alpha$  level were also suppressed following statin treatment in mice (Zeiser, Youssef et al. 2007).

Here, we demonstrated the effect of simvastatin on the production of cytokines such as IL-12p40, IL-6 and TNF- $\alpha$  in IFN- $\gamma$  activated macrophages. We observed that simvastatin significantly enhanced the secretion of IL-12p40 and TNF- $\alpha$  as LM infection progressed in macrophages. This finding is in line with a previous study showing that simvastatin increased LPS-induced IL-12p40 and TNF- $\alpha$  production in peritoneal macrophages by a mechanism involving AP-1 and C/EBP transcription factors. The effect of simvastatin on LPS-induced pro-inflammatory cytokine was mediated by the inhibition of the prenylation pathway, which is important for post-translational modifications. In this study,

the exogenous addition of mevalonate, farnesylpyrophosphate and geranylgeranylprophosphate reversed the statin-mediated effect on IL-12p40. This indicated that the simvastatin-mediated effect on IL-12p40 may be due to inhibition of prenylated proteins rather than depletion of cholesterol (Matsumoto, Einhaus et al. 2004). These data show that the decrease in bacterial growth following statin treatment was associated with minor changes in cytokine and NO levels. Collectively, these results underline that the simvastatin-mediated reduction of LM growth was independent of cytokine production and classical reactive oxygen killing effector mechanisms.

Thus, we investigated activation of macrophages following statin treatment. Simvastatin suppressed expression of MHC-II in macrophages following IFN- $\gamma$  activation, this inhibitory effect was reversed by the addition of exogenous mevalonate. Furthermore, we found that simvastatin has no effect on constitutive expression. Our results show that simvastatin can modulate immune response by decreasing MHC-II expression induced by IFN- $\gamma$  in macrophages. It has been shown that effect of statins on expression of MHC-II in IFN- $\gamma$  activated cells is attributed to the down regulation of inducible class II transactivator promoter IV (CIITA), which in turn lead to reduction in MHC-II expression and thus decreasing T cell activation (Kwak, Mulhaupt et al. 2000). Our results are consistent with previous results showed that statin mediated reduced MHC-II expression was abrogated following mevalonate supplementation (Vaughan, Gotto et al. 2000). Importantly, statins specifically target IFN- $\gamma$  induced expression of MHC-II and has no effect on constitutive expression of MHC-II on human dendritic cells (DC) and B lymphocytes (Pedersen 1999).

Given that statins had a potential role to alter the outcome of infection and to complement our findings with LM infection *in vivo*. In the third part of the study, we investigated the role of statins during *Mtb* infection following aerosol challenge in mice, which closely reflects the natural route of infection in humans. Mice treated with statins for long duration before and after *Mtb* infection, owing to slow growing nature of mycobacterium. In this experiment, we compared antimicrobial potential of three different statins (simvastatin, atorvastatin & rosuvastatin). We noted only simvastatin and atorvastatin showed nearly 1-log



significant decrease in bacterial loads of *Mtb* in spleen, whereas rosuvastatin did not reach significance. In contrast, only rosuvastatin showed significant decrease in growth of *Mtb* in lungs. This discrepancy among statins could be due to the highest half-life (19 hours) of rosuvastatin as compared to simvastatin (3 hours) or atorvastatin (14 hours), which could explain the reduced burden in lungs of rosuvastatin-treated mice. Moreover, lung histopathology revealed smaller microabscesses in statin-treated mice as compared to control, indicating less inflammation. An *in vivo* result in present study shows that statin influences primary infection and dissemination of *Mtb* in mouse model of tuberculosis. To our knowledge, this study provides the first evidence of the beneficial effect of statins on growth of *Mtb in vivo*.

In order to explore mechanism underlying the protective effect of statins, we hypothesized whether statin treatment influences the maturation of phagosomes or autophagy in macrophages. These are the two major pathways which can eliminate intracellular pathogens. Simvastatin-induced reduction in cholesterol in macrophages, counteract phagosomal maturation inhibition, as shown by increased association/colocalization of phagosome containing GFP-expressing *Mtb* with early endosomal antigen 1 (EEA1) followed by the late lysosomal-associated membrane protein 1 (LAMP1). This suggests that statins influences phagosomal maturation, which in turn results in clearance of intracellular *Mtb*. These findings are in line with a previous report showed an increased association of *Salmonella*-containing vacuole (SCV) with the late endosomal marker, cathepsin D (Catron, Lange et al. 2004). One of the main host defense mechanisms against intracellular pathogens is the maturation of phagosomes to phagolysosomes, providing a hostile environment important for efficient killing. Once internalized by macrophages, *Mtb* associate with lipid droplets, which are cholesterol rich depot (Russell, Cardona et al. 2009). Cholesterol is reported to accumulate at endosomes in order to delay and inhibit phagolysosomal maturation (Kondo and Kanai 1976; Manes, del Real et al. 2003). This delay inhibits phagosomal maturation allowing *Mtb* to persist and evade macrophage microbicidal artillery (Clemens and Horwitz 1995; Desjardins and Descoteaux 1997; Westcott, Henry et al. 2010).

As an alternative host defense mechanism, autophagy is induced during infection to limit *L. monocytogenes* (Rich, Burkett et al. 2003) and *M. tuberculosis* growth (Singh, Davis et al. 2006; Py, Lipinski et al. 2007; Deretic 2008). Here, we showed an increased association/co-localization of GFP-expressing *M. tuberculosis* with LC3-II, a marker of autophagosomes that remain on phagosomes even after fusion with lysosomes, following treatment of macrophages with simvastatin. The effect of statin on autophagy is in line with recent report showed induction of autophagy in prostate cancer cell line (PC-3), which is mediated by the inhibition of geranylgeranyl, an intermediate of mevalonate pathway (Parikh, Childress et al. 2010). Here, we demonstrate mechanistically for the first time that blocking cholesterol by simvastatin induces phagosomal maturation as well as autophagy. Hence, counteract *Mtb* evasion strategies involving cholesterol, leading to subsequent more efficient host protection against *Mtb*. The finding was in agreement with report showed that induction of autophagy is critical to eliminate *Mtb* from human and mouse macrophages (Gutierrez, Master et al. 2004; Yuk, Shin et al. 2009). A previous published study has reported the role of autophagy gene variant IRGM-251T in contributing protection from tuberculosis (Intemann, Thye et al. 2009). In addition, autophagy has been shown to play a key role in innate and adaptive defense against other intracellular pathogens (Deretic 2009; Jagannath, Lindsey et al. 2009).

We explored phagosomal maturation or autophagy in macrophages infected with *Mtb*. This could not be investigated in the LM infection model as it escapes quickly from phagosome and rapid proliferation of cytosolic bacteria impairs visualization of the very small number of phagosomal listeria and therefore poses a limitation (de Chastellier and Berche 1994; Portnoy, Auerbuch et al. 2002). In addition, LM can persist in the cytoplasm by forming spacious Listeria-containing Phagosomes (SLAPs), which are autophagosome-like compartments that do not mature, allowing slow bacterial growth within enlarged vesicles that are decorated with an autophagy marker (LC3-II) (Birmingham, Canadien et al. 2007). LM can be internalized by the autophagic vacuole which is then subsequently degraded by lysosomal fusion (Rich, Burkett et al. 2003). However, autophagy mediated clearance of LM is dependent on an intracellular

peptidoglycan-sensing molecule, which triggers autophagy to control LM infection and survival of the host (Zaidman-Remy, Herve et al. 2006; Yano, Mita et al. 2008)). Therefore, phagosomal studies were performed on slow growing *Mtb* bacterium, which arrest maturation of late phagosomes.

It could be speculated that observed reduction in intracellular growth of bacteria might be due to the direct inhibitory effect of statins on HMG-CoA reductase of LM or homologue of *Mtb*. We found no difference in extracellular growth of either *Mtb* or LM in culture broth medium in the presence or absence of statin supplementation. Therefore, it is unlikely that observed reduction in intracellular bacterial growth was a function of direct inhibitory effect of statins on bacterial HMG-CoA reductase. In contrast, studies have reported a direct inhibitory role of statins on fungal growth in culture medium. However, these fungicidal effects were achieved at very high doses of statins, which extrapolated to clinical doses in patients largely exceeding the recommended daily dose allowance (Qiao, Kontoyiannis et al. 2007; Nyilasi, Kocsube et al. 2010; Fowler, Cooper et al. 2011). One possible explanation for the difference between extracellular growth of fungi and bacteria in presence of statins is that prokaryotes utilize class II reductase enzymes and its inhibition requires 1000-fold higher concentrations of statins than compared to class I enzymes utilized by eukaryotes (Hedl and Rodwell 2004).

Recent evidence suggests that *L. monocytogenes* posses its own HMG-CoA reductase, but it is least sensitive to statins. Conflicting evidence suggests that the *Mtb* genome contains an HMG-CoA reductase orthologue (Lamb, Kelly et al. 1998; Bellamine, Mangla et al. 2001). However, other research groups were unable to identify these genes in *Mtb* (Cole, Brosch et al. 1998; Boucher and Doolittle 2000; Bailey, Mahapatra et al. 2002).

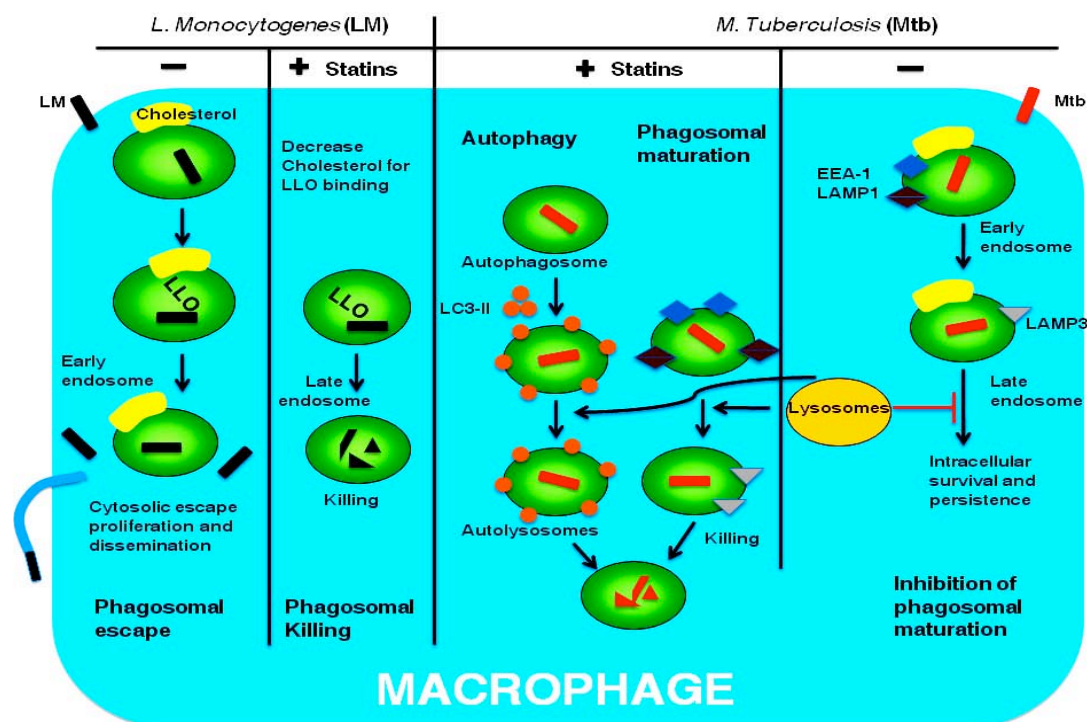
The success of intracellular bacterial pathogens to survive and proliferate in host cells requires multiple adaptation process. One of such process is to adapt their metabolism to harness host nutrients that can be used as alternative carbon source which is critically linked to pathogenesis (Munoz-Elias and McKinney 2006; Brown, Palmer et al. 2008). For instance, LM exploits host cellular glycerol and aromatic amino acids, as a major source for carbon

metabolism but not cholesterol. Host generated fatty acids, glycerol or glycerol-3-phosphate derived from host cellular lipids and phospholipids or triglycerides are the most widely exploited carbon sources by *Mtb* during proliferation in BMDM. The dependency on these carbon substrates particularly fatty acids or cholesterol is further reflected during acute and persistent phase of *Mtb* infection mice (McKinney, Honer zu Bentrup et al. 2000). The identification of homologous mammalian cell entry (mce) operon recently in *Mtb* encode for lipid transporters and metabolism of the imported substrates (Joshi, Pandey et al. 2006; Casali and Riley 2007). The uptake of cholesterol via mce4 operon and catabolism is required for the mycobacterial growth in activated macrophages. Therefore, *Mtb* can use fatty acids and cholesterol as carbon source (Van der Geize, Yam et al. 2007). Furthermore, the deletion in the mce operons showed marked decrease in virulence in mouse model of tuberculosis (Gioffre, Infante et al. 2005).

To explore the importance of our findings in relevance to human subjects, we recruit patients for participation in the study with familial hypercholesterolemia (FH), a genetic mutation of the Low-Density Lipoprotein Receptor (LDLR) gene, situated on chromosome 19 (Hobbs, Russell et al. 1990). This disorder leads to impaired cellular uptake of LDL-cholesterol, which consequently results in increased serum LDL-cholesterol levels. Statins are the first line of drugs for the treatment of patients with hypercholesterolemia. By investigating PBMC from FH patients on statin therapy, we studied effect statins at physiological concentrations (1-15 nmol/L in serum) in human (Linda Björkhem-Bergman 2011), without the solvent (DMSO) that are present in the *in vitro* experiments. Here, we conducted a small pilot-study where PBMC from FH patients significantly reduce mycobacterial growth by 3-fold when compared to PBMC isolated from healthy donors. In addition, growth of *Mtb* was also decreased in monocyte-derived macrophages (MDM) from patients with FH when compared to MDM from healthy donors. Importantly, cellular uptake of *Mtb* was similar in both groups reflecting that reduced growth in FH patients was not due to a defect in initial uptake. It is noteworthy to mention that statins are hepatoselective drugs, preferably metabolized by the liver and PBMC in circulation get exposed to statins to acquire immunomodulatory functions. It has

been reported that statins can inhibit accumulation of cholesterol esters in human MDM (Kempen, Vermeer et al. 1991). This finding emphasizes that statin therapy in patients can induce immunomodulatory properties in PBMC resulting in subsequent reduced growth of *Mtb* in vitro.

Here, we provide evidence that statins can induce host protective functions against *L. monocytogenes* and *M. tuberculosis* infection, which is summarized in Figure 1.



**Figure 1. Proposed model on statin-mediated protection against LM and *Mtb* infection.**

Statins targets LLO-mediated vacuolar escape of LM by limiting the availability of cholesterol required for the binding and activity of LLO and enhanced phagosomal maturation and autophagy, results in clearance of *Mtb* in macrophages by reducing intracellular cholesterol.

In addition, another study from our lab (unpublished, Hartley 2010) shows similar statin-mediated host protective responses in macrophages and mice infected with a protozoan parasite *Leishmania major*. Naturally resistant and susceptible mice strains show reduced parasitemia in footpads and draining lymph nodes following simvastatin treatment compared to control groups. A major finding of this study was that parasite burden in the draining cervical lymph node was decreased following topical application of simvastatin on ear

lesions. These findings suggest a potential use for statins as an ointment in patients with cutaneous leishmaniasis caused by *L. major*. Local application of statins has already been used in the field of osteology, where topical fluvastatin applied at the site of bone implants enhanced the healing process (Ayukawa, Yasukawa et al. 2009; Moriyama, Ayukawa et al. 2010). Similar results were obtained after local injections of simvastatin at the site of bone injury in rats which was attributed, at least in part, to a suppression of macrophage-derived osteoclasts (Ayukawa, Yasukawa et al. 2009), perhaps inferring to their immunomodulatory function.

However, it is important to mention that pharmacological modulation of the cholesterol biosynthesis pathway using statins could offer an advantage. Targeting a rate-limiting enzyme (HMG-CoA reductase) at the beginning of the pathway, as all enzymes in cholesterol biosynthesis pathway are not rate-limiting and therefore their inhibition might not be sufficient to alter prenylation and isoprenoids branch points but sufficient to alter intracellular cholesterol levels. There is also a possibility that residual non rate-limiting enzymes of this pathway are enough to keep it operational (Bengoechea-Alonso and Ericsson 2007). Thereby, increasing specificity and decreasing the impact on other metabolites of the mevalonate pathway. Additional studies by sequentially targeting enzymes/products of the mevalonate pathway are warranted to discover key metabolite exploited by pathogens. These mechanisms need to be fully understood before statins can be used as immunomodulatory agents in the therapy against infectious diseases.

In conclusion, the results presented in this thesis revealed a protective and therapeutic effect of statins in experimental models of infectious diseases such as listeriosis and tuberculosis. Thus, inhibiting the mevalonate pathway by statins can induce host protection against intracellular pathogens reflecting its potential in immunotherapy and adjunctive drug development in infectious diseases.

## References

- Ayukawa, Y., E. Yasukawa, et al. (2009). "Local application of statin promotes bone repair through the suppression of osteoclasts and the enhancement of osteoblasts at bone-healing sites in rats." *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 107(3): 336-342.
- Bailey, A. M., S. Mahapatra, et al. (2002). "Identification, cloning, purification, and enzymatic characterization of Mycobacterium tuberculosis 1-deoxy-D-xylulose 5-phosphate synthase." *Glycobiology* 12(12): 813-820.
- Bellamine, A., A. T. Mangla, et al. (2001). "Structural requirements for substrate recognition of Mycobacterium tuberculosis 14 alpha-demethylase: implications for sterol biosynthesis." *J Lipid Res* 42(1): 128-136.
- Bengoechea-Alonso, M. T. and J. Ericsson (2007). "SREBP in signal transduction: cholesterol metabolism and beyond." *Current Opinion in Cell Biology* 19(2): 215-222.
- Birmingham, C. L., V. Canadien, et al. (2007). "Listeria monocytogenes evades killing by autophagy during colonization of host cells." *Autophagy* 3(5): 442-451.
- Boucher, Y. and W. F. Doolittle (2000). "The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways." *Mol Microbiol* 37(4): 703-716.
- Brown, S. A., K. L. Palmer, et al. (2008). "Revisiting the host as a growth medium." *Nat Rev Microbiol* 6(9): 657-666.
- Casali, N. and L. W. Riley (2007). "A phylogenomic analysis of the Actinomycetales mce operons." *BMC Genomics* 8: 60.
- Catron, D. M., Y. Lange, et al. (2004). "Salmonella enterica serovar Typhimurium requires nonsterol precursors of the cholesterol biosynthetic pathway for intracellular proliferation." *Infection and immunity* 72(2): 1036-1042.
- Cirillo, D. J. (2008). "The effect of statin use on incident immune-mediated and infectious conditions among U.S. veterans." *PhD diss, University of Iowa*.
- Clemens, D. L. and M. A. Horwitz (1995). "Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited." *The Journal of experimental medicine* 181(1): 257-270.
- Cole, S. T., R. Brosch, et al. (1998). "Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence." *Nature* 393(6685): 537-544.
- Coppens, I., A. P. Sinai, et al. (2000). "Toxoplasma gondii exploits host low-density lipoprotein receptor-mediated endocytosis for cholesterol acquisition." *The Journal of cell biology* 149(1): 167-180.
- de Chastellier, C. and P. Berche (1994). "Fate of Listeria monocytogenes in murine macrophages: evidence for simultaneous killing and survival of intracellular bacteria." *Infect Immun* 62(2): 543-553.
- Denoyelle, C., P. Albanese, et al. (2003). "Molecular mechanism of the anti-cancer activity of cerivastatin, an inhibitor of HMG-CoA reductase, on aggressive human breast cancer cells." *Cell Signal* 15(3): 327-338.

- Deretic, V. (2008). "Autophagy, an immunologic magic bullet: Mycobacterium tuberculosis phagosome maturation block and how to bypass it." Future Microbiol 3: 517-524.
- Deretic, V. (2009). "Links between autophagy, innate immunity, inflammation and Crohn's disease." Dig Dis 27(3): 246-251.
- Desjardins, M. and A. Descoteaux (1997). "Inhibition of phagolysosomal biogenesis by the Leishmania lipophosphoglycan." The Journal of experimental medicine 185(12): 2061-2068.
- Djaldetti, M., H. Salman, et al. (2006). "Effect of pravastatin, simvastatin and atorvastatin on the phagocytic activity of mouse peritoneal macrophages." Experimental and molecular pathology 80(2): 160-164.
- Fowler, D. M., S. J. Cooper, et al. (2011). "Suppression of statin effectiveness by copper and zinc in yeast and human cells." Mol Biosyst 7(2): 533-544.
- Gaillard, J. L., P. Berche, et al. (1991). "Entry of L. monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci." Cell 65(7): 1127-1141.
- Gatfield, J. and J. Pieters (2000). "Essential role for cholesterol in entry of mycobacteria into macrophages." Science 288(5471): 1647-1650.
- Gioffre, A., E. Infante, et al. (2005). "Mutation in mce operons attenuates Mycobacterium tuberculosis virulence." Microbes Infect 7(3): 325-334.
- Glynn, S. A., D. O'Sullivan, et al. (2008). "The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, simvastatin, lovastatin and mevastatin inhibit proliferation and invasion of melanoma cells." BMC Cancer 8: 9.
- Gutierrez, M. G., S. S. Master, et al. (2004). "Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages." Cell 119(6): 753-766.
- Hamelin, B. A. and J. Turgeon (1998). "Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors." Trends Pharmacol Sci 19(1): 26-37.
- Hartley, M. A. (2010). "The Immunomodulatory Potential of the Cholesterol-Biosynthesis Pathway in Leishmaniasis." Unpublished.
- Hedl, M. and V. W. Rodwell (2004). "Inhibition of the class II HMG-CoA reductase of Pseudomonas mevalonii." Protein Sci 13(6): 1693-1697.
- Hobbs, H. H., D. W. Russell, et al. (1990). "The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein." Annual Review of Genetics 24: 133-170.
- Intemann, C. D., T. Thye, et al. (2009). "Autophagy gene variant IRGM -261T contributes to protection from tuberculosis caused by Mycobacterium tuberculosis but not by M. africanum strains." PLoS Pathog 5(9): e1000577.
- Itakura, H., T. Kita, et al. (2008). "Relationship between coronary events and serum cholesterol during 10 years of low-dose simvastatin therapy: long-term efficacy and safety in Japanese patients with hypercholesterolemia in the Japan Lipid Intervention Trial (J-LIT) Extension 10 Study, a prospective large-scale observational cohort study." Circ J 72(8): 1218-1224.
- Jagannath, C., D. R. Lindsey, et al. (2009). "Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells." Nat Med 15(3): 267-276.



- Joshi, S. M., A. K. Pandey, et al. (2006). "Characterization of mycobacterial virulence genes through genetic interaction mapping." Proc Natl Acad Sci U S A 103(31): 11760-11765.
- Kempen, H. J., M. Vermeer, et al. (1991). "Vastatins inhibit cholesterol ester accumulation in human monocyte-derived macrophages." Arteriosclerosis and Thrombosis 11(1): 146-153.
- Kikuchi, T., Y. Nagata, et al. (1997). "In vitro and in vivo antiproliferative effects of simvastatin, an HMG-CoA reductase inhibitor, on human glioma cells." Journal of neuro-oncology 34(3): 233-239.
- Kita, T., M. S. Brown, et al. (1980). "Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in livers of mice treated with mevinolin, a competitive inhibitor of the reductase." The Journal of clinical investigation 66(5): 1094-1100.
- Kondo, E. and K. Kanai (1976). "Accumulation of cholesterol esters in macrophages incubated with mycobacteria in vitro." Jpn J Med Sci Biol 29(3): 123-137.
- Krause, B. R. and H. M. Princen (1998). "Lack of predictability of classical animal models for hypolipidemic activity: a good time for mice?" Atherosclerosis 140(1): 15-24.
- Kwak, B., F. Mulhaupt, et al. (2000). "Statins as a newly recognized type of immunomodulator." Nature medicine 6(12): 1399-1402.
- Lamb, D. C., D. E. Kelly, et al. (1998). "A sterol biosynthetic pathway in Mycobacterium." FEBS Lett 437(1-2): 142-144.
- Lecuit, M., S. Vandormael-Pournin, et al. (2001). "A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier." Science 292(5522): 1722-1725.
- Leung, B. P., N. Sattar, et al. (2003). "A novel anti-inflammatory role for simvastatin in inflammatory arthritis." J Immunol 170(3): 1524-1530.
- Liappis, A. P., V. L. Kan, et al. (2001). "The effect of statins on mortality in patients with bacteremia." Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 33(8): 1352-1357.
- Linda Björkhem-Bergman, J. D. L. P. B. (2011). "What is a relevant statin concentration in cell experiments claiming pleiotropic effects?" British Journal of Clinical Pharmacology 72(1): 164-165.
- Loike, J. D., D. Y. Shabtai, et al. (2004). "Statin inhibition of Fc receptor-mediated phagocytosis by macrophages is modulated by cell activation and cholesterol." Arteriosclerosis, thrombosis, and vascular biology 24(11): 2051-2056.
- Manes, S., G. del Real, et al. (2003). "Pathogens: raft hijackers." Nat Rev Immunol 3(7): 557-568.
- Matsumoto, M., D. Einhaus, et al. (2004). "Simvastatin augments lipopolysaccharide-induced proinflammatory responses in macrophages by differential regulation of the c-Fos and c-Jun transcription factors." Journal of immunology 172(12): 7377-7384.
- McKinney, J. D., K. Honer zu Bentrup, et al. (2000). "Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase." Nature 406(6797): 735-738.

- Montero, M. T., O. Hernandez, et al. (2000). "Hydroxymethylglutaryl-coenzyme A reductase inhibition stimulates caspase-1 activity and Th1-cytokine release in peripheral blood mononuclear cells." *Atherosclerosis* 153(2): 303-313.
- Moriyama, Y., Y. Ayukawa, et al. (2010). "Local application of fluvastatin improves peri-implant bone quantity and mechanical properties: a rodent study." *Acta Biomater* 6(4): 1610-1618.
- Mundy, G., R. Garrett, et al. (1999). "Stimulation of bone formation in vitro and in rodents by statins." *Science* 286(5446): 1946-1949.
- Munoz-Elias, E. J. and J. D. McKinney (2006). "Carbon metabolism of intracellular bacteria." *Cell Microbiol* 8(1): 10-22.
- Negre-Aminou, P., A. K. van Vliet, et al. (1997). "Inhibition of proliferation of human smooth muscle cells by various HMG-CoA reductase inhibitors; comparison with other human cell types." *Biochimica et biophysica acta* 1345(3): 259-268.
- Ni, W., K. Egashira, et al. (2001). "Antiinflammatory and antiarteriosclerotic actions of HMG-CoA reductase inhibitors in a rat model of chronic inhibition of nitric oxide synthesis." *Circ Res* 89(5): 415-421.
- Nyilasi, I., S. Kocsube, et al. (2010). "In vitro synergistic interactions of the effects of various statins and azoles against some clinically important fungi." *FEMS Microbiol Lett* 307(2): 175-184.
- Parikh, A., C. Childress, et al. (2010). "Statin-induced autophagy by inhibition of geranylgeranyl biosynthesis in prostate cancer PC3 cells." *Prostate* 70(9): 971-981.
- Parquet, V., S. Briolant, et al. (2009). "Atorvastatin is a promising partner for antimalarial drugs in treatment of Plasmodium falciparum malaria." *Antimicrobial agents and chemotherapy* 53(6): 2248-2252.
- Pedersen, T. R. (1999). "Statin trials and goals of cholesterol-lowering therapy after AML." *Am Heart J* 138(2 Pt 2): S177-182.
- Portnoy, D. A., V. Auerbuch, et al. (2002). "The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity." *The Journal of cell biology* 158(3): 409-414.
- Py, B. F., M. M. Lipinski, et al. (2007). "Autophagy limits Listeria monocytogenes intracellular growth in the early phase of primary infection." *Autophagy* 3(2): 117-125.
- Qiao, J., D. P. Kontoyiannis, et al. (2007). "Antifungal activity of statins against Aspergillus species." *Med Mycol* 45(7): 589-593.
- Rich, K. A., C. Burkett, et al. (2003). "Cytoplasmic bacteria can be targets for autophagy." *Cell Microbiol* 5(7): 455-468.
- Rodal, S. K., G. Skretting, et al. (1999). "Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles." *Mol Biol Cell* 10(4): 961-974.
- Rosch, J. W., A. R. Boyd, et al. (2010). "Statins protect against fulminant pneumococcal infection and cytolysin toxicity in a mouse model of sickle cell disease." *J Clin Invest* 120(2): 627-635.
- Rothwell, C., A. Lebreton, et al. (2009). "Cholesterol biosynthesis modulation regulates dengue viral replication." *Virology* 389(1-2): 8-19.

- Russell, D. G., P. J. Cardona, et al. (2009). "Foamy macrophages and the progression of the human tuberculosis granuloma." *Nat Immunol* 10(9): 943-948.
- Salman, H., M. Bergman, et al. (2008). "Hydrophobic but not hydrophilic statins enhance phagocytosis and decrease apoptosis of human peripheral blood cells in vitro." *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 62(1): 41-45.
- Shepherd, J., S. M. Cobbe, et al. (1995). "Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group." *N Engl J Med* 333(20): 1301-1307.
- Singh, S. B., A. S. Davis, et al. (2006). "Human IRGM induces autophagy to eliminate intracellular mycobacteria." *Science* 313(5792): 1438-1441.
- Soltani, C. E., E. M. Hotze, et al. (2007). "Structural elements of the cholesterol-dependent cytolysins that are responsible for their cholesterol-sensitive membrane interactions." *Proc Natl Acad Sci U S A* 104(51): 20226-20231.
- Thomsen, R. W., H. H. Hundborg, et al. (2006). "Statin use and mortality within 180 days after bacteremia: a population-based cohort study." *Crit Care Med* 34(4): 1080-1086.
- Tleyjeh, I. M., T. Kashour, et al. (2009). "Statins for the prevention and treatment of infections: a systematic review and meta-analysis." *Arch Intern Med* 169(18): 1658-1667.
- Ukomadu, C. and A. Dutta (2003). "p21-dependent inhibition of colon cancer cell growth by mevastatin is independent of inhibition of G1 cyclin-dependent kinases." *J Biol Chem* 278(44): 43586-43594.
- Van der Geize, R., K. Yam, et al. (2007). "A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into Mycobacterium tuberculosis survival in macrophages." *Proc Natl Acad Sci U S A* 104(6): 1947-1952.
- Vaughan, C. J., A. M. Gotto, Jr., et al. (2000). "The evolving role of statins in the management of atherosclerosis." *J Am Coll Cardiol* 35(1): 1-10.
- Weitz-Schmidt, G., K. Welzenbach, et al. (2001). "Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site." *Nat Med* 7(6): 687-692.
- Westcott, M. M., C. J. Henry, et al. (2010). "Dendritic cells inhibit the progression of Listeria monocytogenes intracellular infection by retaining bacteria in major histocompatibility complex class II-rich phagosomes and by limiting cytosolic growth." *Infection and immunity* 78(7): 2956-2965.
- Yano, T., S. Mita, et al. (2008). "Autophagic control of listeria through intracellular innate immune recognition in drosophila." *Nat Immunol* 9(8): 908-916.
- Yilmaz, A., C. Reiss, et al. (2006). "Differential effects of statins on relevant functions of human monocyte-derived dendritic cells." *J Leukoc Biol* 79(3): 529-538.
- Yuk, J. M., D. M. Shin, et al. (2009). "Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin." *Cell Host Microbe* 6(3): 231-243.

- Zaidman-Remy, A., M. Herve, et al. (2006). "The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection." *Immunity* 24(4): 463-473.
- Zeiser, R., S. Youssef, et al. (2007). "Preemptive HMG-CoA reductase inhibition provides graft-versus-host disease protection by Th-2 polarization while sparing graft-versus-leukemia activity." *Blood* 110(13): 4588-4598.

University of Cape Town

# **Chapter 5**

## **Future Prospective**

University of Cape Town

## 5. Future Prospective

Considering the promising results from this study we aim to use statins to screen for potential new drug targets in the cholesterol biosynthesis pathway that may act as novel therapy against infectious diseases.

### Rationale

Targeting host HMG-CoA reductase at the proximal end with statins has potential to uncover the role of other intermediates from branch points of the pathway to identify new drug targets.

### Objectives

(A) To identify host defence mechanisms between untreated and statin-treated peripheral blood mononuclear cells from patients with active TB, statin-treated patients with hypercholesterolemia and healthy controls.

(B) To determine gene and protein expression profiles between untreated and statin-treated human monocytic cell lines to *Mtb* infection using microarray and proteomics.

(C) To functionally evaluate target genes by siRNA and inhibitor screening using chemical analogues in human monocytic cell lines or primary cells infected with *Mtb*. The specificity and affinity of the target genes will be further investigated using chemical analogues.

### Significance

The study will provide a better understanding of the host cholesterol biosynthesis pathway. This will intensify the search for novel host-directed drug targets against tuberculosis.

### Novelty

The approach of using well tolerated drugs in humans such as statins, which has no direct effect on bacterial reductase itself are likely to overcome the limitation of widely used antibiotics which often result in drug-resistance.